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Direct doping analysis of beta-blocker drugs from urinary samples by online molecularly imprinted solid-phase extraction coupled to liquid chromatography/mass spectrometry

Mariane Gonçalves Santos, Isabela Campos Tavares, Vanessa Bergamin Boralli, Eduardo Costa Figueiredo*

Toxicants and Drugs Analysis Laboratory - LATF, Faculty of Pharmaceutical Sciences, Federal University of Alfenas - Unifal-MG, 700 Gabriel Monteiro da Silva street, 37130-000, Alfenas, MG, Brazil

*Corresponding author: Tel.: +55 35 3299 1342; Fax: +55 35 3299 1067

E-mail: eduardocfig@yahoo.com.br

Abstract

The use of beta-blockers to enhance performance in some sports is forbidden. Based on this regulation, there is a demand for dynamic analytical procedures for analyzing these compounds quickly and without manual sample preparation. Therefore, the use of a molecularly imprinted polymer (MIP) in a multidimensional liquid chromatographic system coupled to a mass spectrometer provides a good alternative for improving the selectivity and practicality of the beta-blockers analyses, as described in this paper. A water-compatible MIP for oxprenolol was synthesized by the precipitation method, using methacrylic acid as a functional monomer and 2-hydroxyethyl methacrylate and glycerol dimethacrylate as hydrophilic monomers. A column filled with MIP was coupled to an LC-MS/MS instrument under multidimensional configuration, with 10.0 mmol L^{-1} ammonium formate buffer (pH 5.0) as the loading and reconditioning mobile phase and a 0.01% formic acid aqueous solution:methanol (30:70 v:v) as the elution mobile phase. The system was used for on-line extraction and quantization of oxprenolol (from 1.0 to 75.0 µg L^{-1}), atenolol, propranolol, nadolol, pindolol, labetalol and metoprolol (all from 3.0 to 50 µg L^{-1}) simultaneously, from urine samples. The correlation coefficient was higher than 0.99 for all of the analytes. Suitable precision and accuracy were obtained.

Keywords: doping, mass spectrometer, MIP, beta-blockers, column switching, direct analysis.

Introduction

Beta-blockers are drugs commonly used in angina pectoris and hypertension treatments [1]. The drugs are classified as β1 or β2 adrenergic antagonists and act by relaxing muscles and reducing the heart rate [2]. For these reasons, these compounds can be used to improve performance in sports that require accurate steadiness, equilibrium and deftness, such as archery, shooting, gymnastics, golf, darts, automobile racing and billiards [2,3,4]. Fig. 1 shows the key beta-blockers used for some sports that are forbidden by the World Anti-Doping Agency (WADA, 2014).

Doping control for these compounds is commonly carried out by analyzing their presence in urine samples by gas chromatography/mass spectrometry or liquid chromatography/mass spectrometry [1]. However, due to the complexity of urine, sample preparation strategies involving solid-phase extraction [5,6], liquid-liquid extraction [7-9] or pre-column clean-up [10,11] must be used to eliminate interferents and to concentrate the analytes. However, due to the low selectivity of these conventional sample preparation techniques, the use of selective sorbents based on molecularly imprinting technology has been prominent in recent years [12-15] and some applications for beta-blockers can also be found [12,13].

Molecularly imprinted polymers (MIPs) are synthetic materials obtained via the copolymerization of a functional monomer with a cross-linker in the presence of a template molecule. After polymerization, the polymer is washed to eliminate the template molecules, and the obtained binding sites are able to recognize the template in terms of size, shape and chemical functionality [16-20]. For the extraction of analytes from aqueous samples, the molecular recognition can be highly perturbed by the presence of water molecules, because water can establish non-selective bonds

with MIPs recognition sites, thus decreasing the method's selectivity. One strategy that can be used to avoid this behavior is to coat MIPs with hydrophilic groups using hydrophilic monomers added at the end of the polymerization [13,19,21,22]. This hydrophilic layer forms hydrogen bonds with water, thus minimizing the interference of this solvent in the analyte-polymer complex.

Due to the importance of the doping analysis of beta-blockers and the relevant characteristics of MIPs, such as selectivity, sensitivity, high stability, high lifetime, low cost and proper recognition in aqueous matrices, we proposed a new hydrophilic MIP selective for beta-blockers and that can be used in a multidimensional, molecularly imprinted, solid-phase extraction system coupled to a liquid chromatography-tandem mass spectrometry (LC-MS/MS) instrument.

Experimental

Chemicals and solutions

The organic solvents used in this study, namely acetonitrile and methanol, were obtained from Vetec (Rio de Janeiro, Brazil). The solutions were prepared with deionized water (18.2 MΩcm) obtained from a Milli-Q water purification system (Millipore, Bedford, USA). For the MIP synthesis, oxprenolol, methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), and 2,2'-azobisisobutyronitrile (AIBN) were used as the template, functional monomer, crosslinking reagent and initiator, respectively (all from Sigma-Aldrich, Steinheim, Germany). HPLC-grade acetonitrile was used as the solvent. Glycerol dimethacrylate (GDMA) and hydroxymethyl methacrylate (HEMA) (both from Sigma-Aldrich, Steinheim, Germany) were used as hydrophilic co-

monomers. Methanol and acetic acid (Merck, Darmstadt, Germany) were used during the polymer washing steps.

Stock solutions of oxprenolol (OXP), atenolol (ATE), metoprolol (MET), labetalol (LAB), propranolol (PROP), nadolol (NAD) and pindolol (PIN) (all from Sigma-Aldrich, Steinheim, Germany) were prepared at a concentration of 1.0 mg L^{-1} in HPLC-grade methanol, placed in amber flasks and stored at -18.0 °C for up to 30 days. Working solutions of 0.1 to 500.0 µg L^{-1} were prepared daily by diluting stock solutions in methanol, drying the standard solutions under nitrogen flow and resuspending the standards in a corresponding volume of blank sample of human urine. This urine was obtained from voluntaries who agreed to participate in this study. In order to confirm the absence of beta-blockers, the urine was previously tested by the developed method.

Methanol, formic acid (Biotec, Londrina, Brazil), ammonium formate (Fluka, Seelze, Germany), ammonium hydroxide (Isofar, Jacaré, Brazil) and ammonium chloride (Vetec, Rio de Janeiro, Brazil) were used to prepare the mobile phases for LC-MS/MS analysis.

MIP synthesis and characterization

The synthesis of the OXP molecularly imprinted polymer was based on non-covalent interactions between the template and the functional monomer. The synthesis was carried out by the precipitation method (using a large volume of solvent). In a 250.0 mL glass flask, 1.0 mmol of OXP and 4.0 mmol of MAA were dissolved in 24.0 mL of acetonitrile. Afterward, 7.0 mmol of EGDMA and 25.0 mg of AIBN were added, and the mixture was purged with nitrogen for 20 min. The flask was

closed and immersed in a glycerin bath with agitation at 65.0°C. After 1 h of synthesis, a mixture of 7.5 mmol of HEMA, 0.5 mmol of GDMA and 24.0 mL of acetonitrile (previously purged with nitrogen for 20.0 min) was added into the synthesis flask, and the polymerization reaction was carried out for more than 23 h.

MIP particles ranging from 75.0 to 106.0 μm in size were selected using steel sieves, and approximately 500.0 mg was washed in an ultrasonic bath with 10.0 mL of a 9:1 (v:v) methanol:acetic acid solution for 1 h. The washing procedure was repeated 10 times, and the washing solution was renewed for each repetition. After drying at 70.0 °C for 24 h, approximately 70.0 mg of MIP was packed in a steel column (empty HPLC pre-column) and coupled to the column switching system. The non-imprinted polymer (NIP) was synthesized similarly to the MIP but in the absence of the template molecule.

Initially, the materials were characterized by scanning electron microscopy (Zeiss LEO 440, Cambridge, England).

A kinetics study was carried out by adding 10.0 mg of the polymer (MIP or NIP) to test glass tubes containing 1.0 mL of 1.0 mg L^{-1} OXP phosphate buffer solution (0.01 mol L^{-1}, pH 7.0). The tubes were shaken for 0, 15.0, 30.0, 45.0, 60.0, 75.0, 90.0 or 105.0 min at room temperature (approximately 25.0°C) and centrifuged at 1,000×*g*. The OXP remaining in the supernatant was quantified using a system equipped with an HPLC pump coupled to a UV detector (λ: 220 nm, flow rate: 1.0 mL min^{-1}, mobile phase: phosphate buffer solution 0.01 mol L^{-1}, pH 7.0).

Adsorption isotherms were constructed to evaluate the extraction capacities of the MIP and NIP. OXP standard solutions (25.0, 50.0, 100.0, 200.0, 300.0, 500.0, 1000.0 and 1500.0 mg L^{-1}) were prepared in a phosphate buffer solution (0.01 mol L^{-1}, pH 7.0).

One milliliter of each solution was transferred to a glass tube containing 10.0 mg of MIP or NIP, and the tubes were shaken for 60.0 min at room temperature. Afterward, each sample was centrifuged at 1,000×*g* for 10.0 min. The OXP concentration remaining in the supernatant (equilibrium concentration - Ce) was quantified using the above-described system. The mass retained by the polymers (adsorption capacity - Qe) was calculated by subtraction. The results were analyzed by Freundlich and Langmuir models, with the accepted adequacy standard being the linear correlation coefficient (r).

Liquid chromatography and mass spectrometry conditions

LC-MS/MS analyses were performed using an LC-MS 8030 from the Shimadzu® LC-MS 8083 equipment (Kyoto, Japan), equipped with a Shim-Pack XR-ODS C18 (100 x 3 mm, 2.2 µm) chromatographic column and a triple-quadrupole mass analyzer. The positive electrospray ionization mode was selected, and the MRM (Multiple Reaction Monitoring) transitions and optimal collision energies were optimized for each analyte (Table 1). The identification criterion was the simultaneous presence of the three fragments of each molecule, according with Table 1. The quantitative analyses were carried out using the TIC (total ion chromatogram) of the three MRM transitions of each molecule. The oven, interface and heat block temperatures were set to 40.0, 250.0 and 400.0°C, respectively. The nebulizing and drying gas flow rates were 1.5 and 15.0 mL min^{-1}, respectively. The mobile phase of the of the chromatographic column was composed of 0.01% formic acid aqueous solution:methanol at 30:70: (v/v) steps. The flow rate was 0.4 mL min^{-1}. The volume of the sample loop was 100.0 µL, and data files were acquired using the LabSolutions® software program.

Column switching

The column switching system (Fig. 2) consisted of a pump (P) delivering either 10 mmol ammonium formate buffer at pH 5.0 as mobile phase A (MPA) or a 0.01% formic acid aqueous solution:methanol at 30:70 (v/v): as mobile phase B (MPB), both at 0.4 mL min^{-1}. The pump was connected to an autosampler (AS) equipped with an injection valve (V1) with a 100.0 µL loop. The autosampler was connected to an electronic six-port switching valve (V2), and the MIP column (kept at 45.0°C) was positioned between V1 and V2. A C18 analytical column (kept at 25.0°C) was positioned after V2 and before the mass spectrometer (MS). Each cycle of the analysis was composed of three steps. First, 100.0 µL of sample was collected in the loop (Fig. 2A), whereas the MPA flows through the MIP column and no mobile phase flows through the analytical column. Next, valve V1 was switched, and the sample was conducted through the MIP column by MPA for 3.0 min (Fig. 2B), whereas no mobile phase flows through the analytical column. The beta-blockers were retained in the MIP column, and the interferents were eliminated. In the third step, V2 was switched, and MPB eluted the beta-blockers from the MIP and led them to the analytical column and mass spectrometer (Fig. 2C). After 8.5 min, the system returned to the first stage (Fig. 2A), and reconditioning was carried out for 3.5 min. The total time required for each analysis, including the extraction and chromatography/mass spectrometer analysis, was 13.0 min.

Sample preparation

The human urine sample handling procedure was approved by the ethics committee of the Federal University of Alfenas (registration number 193.678). The

blank samples were collected from voluntaries who agreed to participate in this study. In order to check the absence of beta-blockers, the samples were previously tested by developed method.

The pH of the samples was previously adjusted to 5.0 using a 1.0 mol L^{-1} formic acid solution. The samples were then centrifuged for 10 min at 1000x*g*, the supernatant was collected and directly analyzed by the column switching system.

Validation study

The linearity, sensitivity, selectivity, precision, accuracy, stability, detection and quantification limits and matrix effect were evaluated. These studies were performed using a pool of blank human urine samples (free of the analytes) spiked with ATE, MET, LAB, PROP, NAD and PIN at concentrations ranging from 1.0 to 50.0 µg L^{-1} and with OXP at concentrations ranging from 0.1 to 75.0 µg L^{-1}. The linearity and sensitivity, which are expressed as the correlation coefficient (r) and the slope of the calibration curve, respectively, were established using three calibration curves (in six replicates) of all of the analytes at six different concentration levels (3.0, 10.0, 20.0, 30.0, 40.0, and 50.0 µg L^{-1} for ATE, MET, LAB, PROP, NAD, and PIN and 1.0, 5.0, 10.0, 25.0, 50.0 and 75.0 µg L^{-1} for OXP). The selectivity was evaluated by observing the presence or absence of peaks at the same retention times of analytes in a blank sample. The intra-assay precision and accuracy were assessed with six replicates at three concentration levels (3.0, 20.0 and 50.0 µg L^{-1} for ATE, MET, LAB, PROP, NAD and PIN and 1.0, 25.0 and 50.0 µg L^{-1} for OXP) on the same day. The inter-assay precision and accuracy were evaluated using six replicates analyzed at three concentration levels (3.0, 20.0 and 50.0 µg L^{-1} for ATE, MET, LAB, PROP, NAD and PIN and 1.0, 25.0 and 50.0 µg L^{-1} for OXP) on

three different days. The results were expressed as relative standard deviation percentages and relative errors for precision and accuracy percentages, respectively. The precision and accuracy test were appraised (evaluated)under the same conditions: equipments, , analyst and laboratory. Only the day was the variable focused. The limits of detection (LOD) and quantification (LOQ) were established based on the signal/noise ratio (three times for LOD and 10 times for LOQ). Stability studies (expressed as a percentage of relative standard deviations) were conducted by analyzing samples at three concentration levels (3.0, 20.0 and 50.0 μg L^{-1} for ATE, MET, LAB, PROP, NAD and PIN and 1.0, 25.0 and 50.0 μg L^{-1} for OXP) after 30 days in a freezer (-18.0°C) and after cycles of freezing and thawing (four cycles of 24 h for each one). The matrix effect was evaluated by analyzing six blank samples from six different individuals (voluntaries), fortified with the analytes at a concentration of 10.0 μg L^{-1}, and the result was expressed as the percentage of relative standard deviation. The absence of matrix effect was considered for a relative standard deviation lower than 15%.

Results and discussion

Polymer characterization

Scanning electron microscopy images of the materials (Fig. 3) revealed a MIP morphological structure presenting microsphere agglomerates due to the precipitation method used for the synthesis.

Adsorption studies were performed for the MIP and NIP. The first investigation examined the MIP adsorption kinetics. It was observed that adsorption equilibrium was reached in 60.0 min (Fig. 4). Then, adsorption isotherms were constructed for both

materials, as described in the section "*MIP synthesis and characterization*". The MIP and NIP were combined with different concentrations of OXP for 60.0 min because this was the time required to reach equilibrium. As shown in Fig. 5, the adsorption showed a linear relationship until equilibrium was reached; the concentrations were approximately 100.0 and 200.0 mg L^{-1}, respectively, for the MIP and NIP. Based on molecular recognition, the MIP presented the highest adsorption capacity, likely due to OXP interactions, whereas only nonspecific interactions prevailed between the NIP and OXP [23,24].

Then, the Freundlich and Langmuir models were tested for the MIP and NIP. Fig. 6 shows that the Langmuir model was the best fit for the MIP and NIP, with correlation coefficients of 0.99 and 0.98, respectively, whereas the Freundlich model's correlation coefficients were 0.96 and 0.92, respectively. The maximum adsorption capacities for the materials were calculated as the inverse of the slope; for the MIP, the capacity was 82.6 mg g^{-1}, and for the NIP, the capacity was 67.1 mg g^{-1}. The Langmuir model indicates that molecules have a uniform distribution in the binding sites around the polymer and that each binding site is able to receive only one molecule. Furthermore, according to the model, the analytes are retained in a monolayer on the MIP surface, and the energy involved in this process is the same for all binding sites surrounding the polymer [25-27].

Method optimization

System optimization was performed using a pool of blank human urine samples spiked with ATE, MET, LAB, PROP, NAD, PIN and OXP at a concentration of 100.0 µg L^{-1}. The following variables were evaluated in a univariate manner: standard/sample pH,

extraction time, mobile phase flow rate and MPA and MPB compositions. The initial conditions were as follows: standard/sample without pH adjustment, extraction time of 1.0 min, mobile phase flow rate of 0.1 mL min^{-1} and water and methanol as MPA and MPB, respectively.

Initially, the standard/sample pH was evaluated from 3.5 to 10.0 using 0.1 mol L^{-1} formic acid or 0.1mol L^{-1} ammonium hydroxide aqueous solutions for adjustment. The best results, expressed as the absolute response of the analytical signal and lower RSD%, were achieved at a pH value of 5.0. At this pH, the analytes and the functional monomer (pKa of approx. 9.0 and 4.0, respectively) were completely ionized, favoring electrostatic interactions between the two [28].

The extraction time can be defined as the time necessary to extract the analytes and to eliminate the interferents (Fig 2A) by passing MPA. In this work, the extraction time ranged from 1.0 to 5.0 min. The best result evaluated for sensitivity and precision was obtained at 3.0 min. Extraction for less than 3.0 min resulted in low sensitivity, which was likely due to insufficient time to prepare the sorbent, to receive the sample and to eliminate interferents. Times longer than 3.0 min resulted in low sensitivity due to the removal of the analytes by the conditioning solution.

The mobile phase flow rate is correlated with the peak symmetry and time retention, and for this reason, this parameter was evaluated from 0.1 to 0.4 mL min^{-1}. The best result was obtained at a flow rate of 0.4 mL min^{-1}, with a decrease in peak broadening and an increase in the analytical frequency. Higher flow rates were tested, but the results were not applicable due to problems with the system pressure.

The properties of MPA are fundamental to improving the extraction of the analytes and eliminating interferents. Thus, water, 10.0 mmol L^{-1} ammonium formate

buffer (pH 4.8), 10.0 mmol L^{-1} ammonium acetate buffer (pH 3.0) and 10.0 mmol L^{-1} ammonia/ammonium buffer (pH 10.0) were evaluated. The best results, expressed as the response of the analytical signal, were obtained by using a 10.0 mmol L^{-1} formate buffer. Peak broadening was also observed for the ammonia/ammonium buffer because beta-blockers are not ionized at this pH. This fact complicates the interaction between the polymer and the analytes. The selected pH was 4.8 because electrostatic interactions between the analytes and the MIP prevailed in this condition, considering their pKa values of approximately 9.0 and 4.0, respectively [28]. Finally, the ammonium formate buffer concentration was studied from 2.0 to 10.0 mmol L^{-1} to determine the concentration at which the ionic strength can affect the analyte ionization processes [29,30]. Higher analytical signals were reached at 10.0 mmol L^{-1} because higher ionic strength improves analyte ionization when the electrospray mode is employed [29]. Higher concentrations were not tested because crystallization in the ion source could occur.

MPB has at least three important functions: to elute the analytes from the MIP column, to separate them in the analytical column and to improve their ionization efficiency in the electrospray source [31]. Additionally, this solution needs to be effective to clean the system after the extraction procedure, thus eliminating the memory effect (carry over). Pure methanol and 0.01% formic acid aqueous solution:methanol combined in proportions of 10:90, 20:80, 30:70 and 40:60 (v:v) were tested as MPB. Employing pure methanol and 40 % of 0.01% formic acid aqueous solution, the analytes were not completely eluted, and the carry over effect was observed. Quantitative elutions were obtained using solutions containing 30.0, 20.0

and 10.0% of 0.01% formic acid aqueous solution, the first of which was selected as the working solution to save the reagents.

After the optimization process, the total time per analysis was 13.05 min. Moreover, the analytical signals were increased in about 884.0, 534.0, 234.0, 235.0, 818.0, 535.0 and 406.0% for ATE, MET, LAB, PROP, NAD, PIN and OXP, respectively, relative to the initial conditions. Fig. 7 shows the chromatograms obtained with the optimized system for a blank urine sample and a blank urine sample fortified with 50.0 μg L^{-1} ATE, MET, LAB, PROP, NAD and PIN and 75.0 μg L^{-1} OXP.

Validation assays and method application

The developed method was linear from 3.0 to 50.0 μg L^{-1} for ATE, MET, LAB, PROP, NAD and PIN and from 1.0 to 75.0 μg L^{-1} for OXP. The intra-assay and inter-assay precisions (obtained as a relative standard deviation) were lower than 20.0% for concentrations near the LOQ and 15.0% for the others, according to the validation guidelines of the United States Food and Drug Administration [32], as shown in Table 2. Good results with respect to accuracy, stability and the matrix effect can be verified in Table 2 as well. Matrix effect was evaluated by using the relative standard deviation between the samples, considering as acceptation criteria values lower than 15.0%. The LODs were 1.0 μg L^{-1} for ATE, MET, LAB, PROP, NAD and PIN and 0.1 μg L^{-1} for OXP, which are appropriate for doping analysis considering the MRPL (Minimum Required Performance Levels) for detection and identification of non-threshold substances [36]. Furthermore, the LODs established in this paper are lower or closer than other values described in literatures as can be seen in table 3. The method was selective since was not observed picks in the blank sample at same retention time of analytes. It should be

emphasized that the same MIP column was used during all of the validation assays, and non-significant differences were observed in the analytical signal after approximately 150 cycles. Additionally, the LOD and LOQ for OXP (template) were lower than those of other beta-blockers, likely due to the more favorable interactions between this molecule and the MIP binding sites.

Fortified human urine samples (at 5.0, 15.0, 35.0 and 45.0 μg L^{-1}) were analyzed by the proposed method, and the results showed a low variation between the nominal concentration and the analyzed concentration, with the relative error ranging from −9.5 to 5.9%.

Conclusions

The developed online system for direct extraction and analysis of beta-blocker drugs in human urine samples was linear for ATE, MET, LAB, PROP, NAD, PIN and OXP and appropriate for doping analysis. Good figures of merit were attained, such as low LOD, wide linear range, good precision and accuracy, high analytical frequency and minimal sample manipulation. Additionally, other interesting system characteristics, such as high selectivity, high MIP column lifetime, use of small sample and solvent volumes, and ease of operation, among others, should be emphasized. Furthermore, it is important to note that the present system could be used to analyze other classes of drugs from biological samples.

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Tables

Table 1: Analytes and their precursors, fragments and collision energies.

Analyte	Precursor (*m/z*)	Fragments (*m/z*)	Collision Energy (kV)
ATE	267.2	145.0	-30
		190.1	-20
		116.1	-10
LAB	329.2	91.1	-35
		162.1	-25
		294.1	-20
NAD	310.1	254.2	-20
		74.1	-25
		201.1	-25
PIN	249.2	116.1	-20
		74.1	-25
		72.1	-25
MET	268.2	116.5	-25
		98.1	-25
		133.2	-25
OXP	266.2	72.1	-25
		224.9	-15
		116.1	-20
PROP	260.2	116.1	-20
		98.1	-20
		183.2	-20

Table 2: Validation parameters of online extraction applied in the LC-MS/MS method for the determination of ATE, MET, LAB, PROP, NAD, PIN and OXP in urine.

Validation parameters	ATE	MET	LAB	PROP	NAD	OXP	PIN
Linear range (µg L^{-1})	3.0-50.0	3.0-50.0	3.0-50.0	3.0-50.0	3.0-50.0	1.0-75.0	3.0-50.0
Linearity (r^2) ($\bar{x}$, n=3)	0.99	0.99	0.99	0.99	0.99	0.99	0.99
Slope (a) ($\bar{x}$, n=3)	3197.8	3316.9	10233.6	3736.4	3739.2	29580.5	7729.4
Intercept (b)	6738.3	1615.7	13674.7	718.4	2953.7	18371.3	8454.0
LOD µg L^{-1}	1.0	1.0	1.0	1.0	1.0	0.1	1.0
LOQ µg L^{-1}	3.0	3.0	3.0	3.0	3.0	1.0	3.0
Matrix effect % RSD (10 µg L^{-1}, n=6)	12.9	11.2	13.5	12.5	9.5	11.1	11.7
Intra-assay precision % RSD (n=6)	7.6[a] 14.4[c] 8.7[e]	14.1[a] 14.9[c] 11.9[e]	13.3[a] 7.6[c] 5.0[e]	14.2[a] 10.8[c] 10.6[e]	19.6[a] 9.7[c] 5.2[e]	13.0[b] 11.0[d] 6.5[e]	11.4[a] 13.2[c] 13.3[e]
Inter-assay precision % RSD (n=6, three days)	12.2[a] 5.6[c] 4.7[e]	13.3[a] 8.5[c] 7.8[e]	10.7[a] 9.9 [c] 9.4[e]	4.6[a] 8.5[c] 11.2[e]	17.4[a] 5.9[c] 8.0[e]	16.9[b] 7.2[d] 11.4[e]	6.5[a] 11.7[c] 5.2[e]
Accuracy E% (n=6)	9.4[a] 12.5[c] -0.8[e]	2.8[a] 12.5[c] -0.6[e]	17.0[a] 2.5[c] -1.9[e]	8.0[a] -2.6[c] 2.5[e]	-12.6[a] 9.8[c] -4.8[e]	-4.5[b] -7.3[d] 4.2[e]	-9.4[a] -4.7[c] -4.1[e]
Accuracy E% (n=6, three days)	-18.0[a] 7.5[c] -9.8[e]	9.5[a] 4.9[c] -3.3[e]	15.3[a] -2.[c] -8.7[e]	-7.5[a] -10.0[c] -9.3[e]	14.1[a] 8.6[c] 7.9[e]	15.0[b] -11.4[d] 5.6[e]	16.2[a] 6.1[c] 8.0[e]
Stability[x] %RSD (n=6)	9.3[a] 10.0[c] 12.5[e]	9.7[a] 12.3[c] 11.9[e]	11.7[a] 13.9[c] 12.2[e]	13.2[a] 8.9[c] 10.4[e]	11.3[a] 13.6[c] 13.3[e]	6.2[b] 5.7[d] 6.0[e]	13.7[a] 10.8[c] 14.1[e]
Stability[y] %RSD (n=6)	10.9[a] 8.8[c] 9.7[e]	14.6[a] 8.1[c] 4.8[e]	14.3[a] 14.5[c] 14.8[e]	10.9[a] 14.7[c] 12.5[e]	7.6[a] 9.0[c] 11.5[e]	5.9[e] 11.6[d] 9.7[e]	10.9[a] 10.8[c] 11.1[e]

[a]3.0 µg L^{-1}, [b]1.0 µg L^{-1}, [c]20.0 µg L^{-1}, [d]25.0 µg L^{-1} and [e]50.0 µg L^{-1}. [x]after 30 days in freezer and [y]after four cycles of freeze and thawing.

Table 3: Comparison between LOD values found in literatures and those obtained in this paper.

Reference	LOD
P. V. Eenoo, W. V. Gansbeke, N. De Brabanter, K. Deventer, F. T. Delbeke, *J. Chromatogr. A*, 2011, **1218**, 3306–3316 [33].	Values between 25.0 and 500.0 µg L^{-1}
M. Kolmonen, A. Leinonen, A. Pelander, I Ojanperä, *Anal. Chim. Acta*, 2007, **585**, 94-102 [34].	500.0 µg L^{-1}
G. J. Murray, J. P. Danaceau. *J. Chromatogr. B*, 2009, **877**, 3857–3864 [2].	Values between 8.0 and 62.0 µg L^{-1}
M-J. Paik, J. Leeb, K-R. Kimb, *Anal. Chim. Acta*, 2007, **601**, 230-233 [35].	Values between 0.03 and 2.7 µg L^{-1}
This paper	Values between 0.1 and 1.0 µg L^{-1}

Figure captions

Fig. 1. Molecular structure of common beta-blockers that are forbidden in some sports by the World Anti-Doping Agency (WADA, 2014).

Fig. 2. Chromatographic system composed of pump (P), sample flask (S), autosampler (AS), injection valve (V1), electronic six-port valve (V2), loop (L), MIP and analytical columns, detector (MS), waste (W). MPA and MPB are the extraction and elution mobile phases, repectively.

Fig. **3.** Scanning electron micrograph of MIP. (A) Magnification of ×6000 and (B) magnification of ×25000.

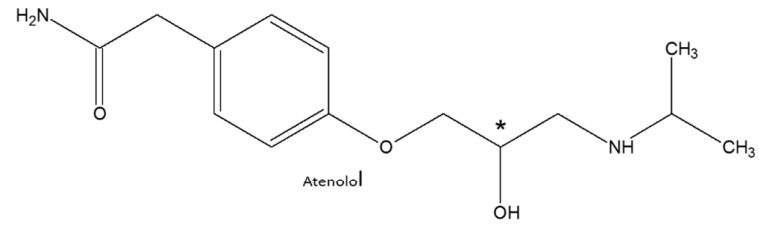
Fig. 4. Absorption kinetics for the MIP and the NIP.

Fig. 5. Adsorption isotherms of OXP for the MIP and the NIP.

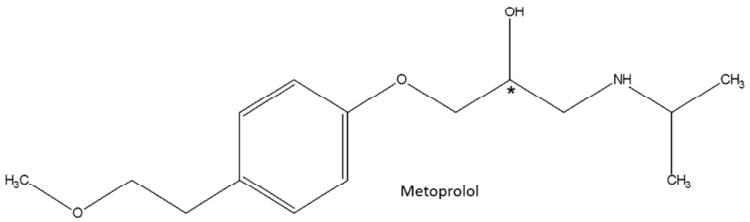
Fig. 6. Langmuir (A) and Freundlich (B) adsorption isotherm of OXP for the MIP and the NIP.

Fig. 7. (A) Chromatograms (TIC) obtained for blank urine sample, (B) after optimization for 1.0 μg L^{-1} 1-ATE, 2-MET, 3-LAB, 4-PROP, 5-NAD and 6-PIN and 0.1 μg L^{-1} for OXP and (C) after optimization for 50.0 μg L^{-1} 1-ATE, 2-MET, 3-LAB, 4-PROP, 5-NAD and 6-PIN and for 75.0 μg L^{-1} 7-OXP.

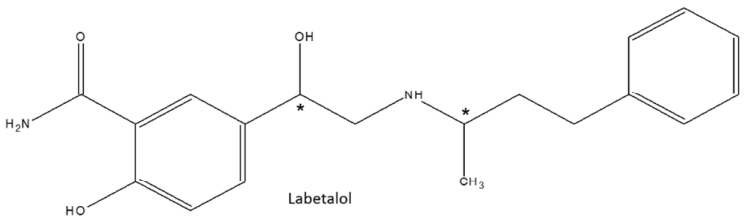
Figure 1



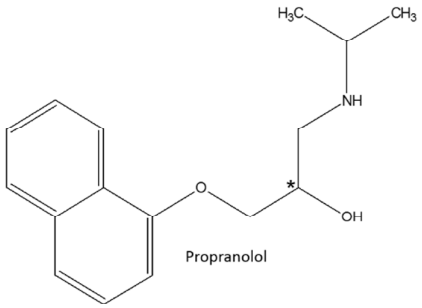
Atenolol



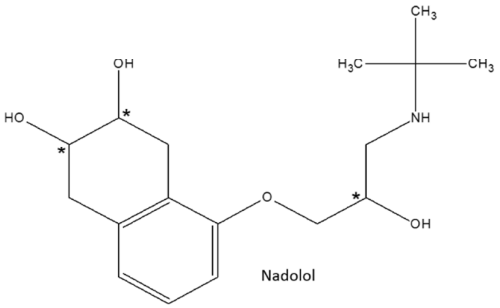
Metoprolol



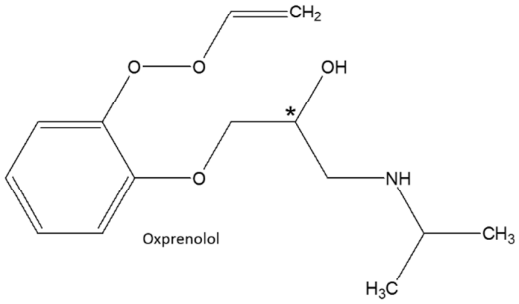
Labetalol



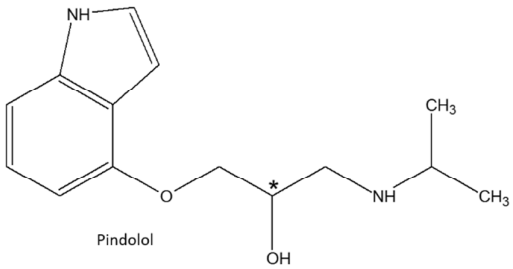
Propranolol



Nadolol



Oxprenolol



Pindolol

* Chiral center

Figure 2

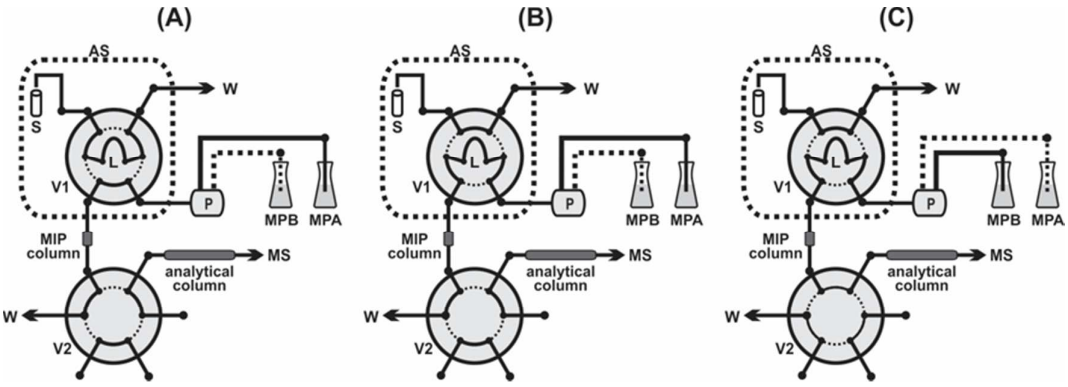


Figure 3

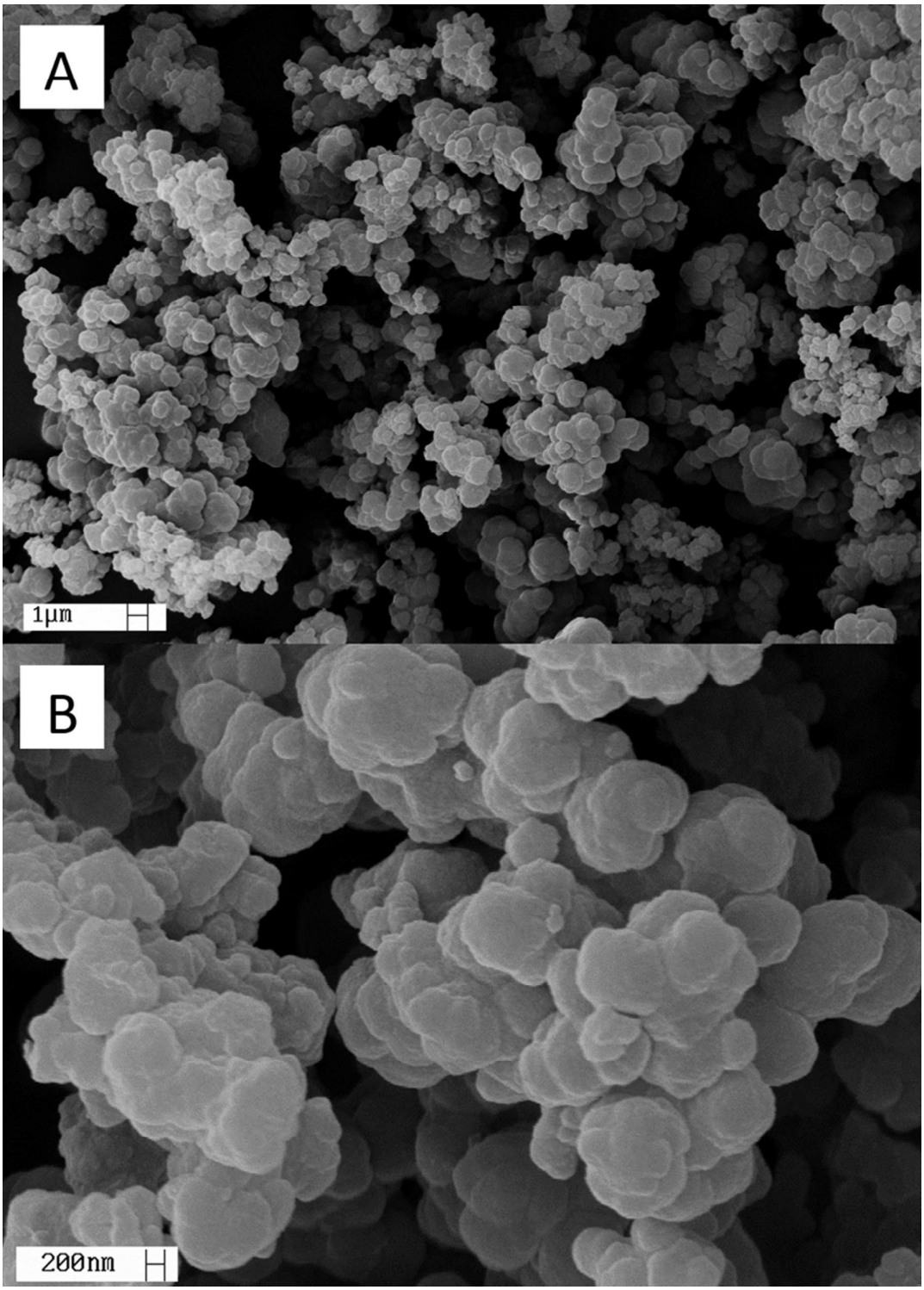


Figure 4

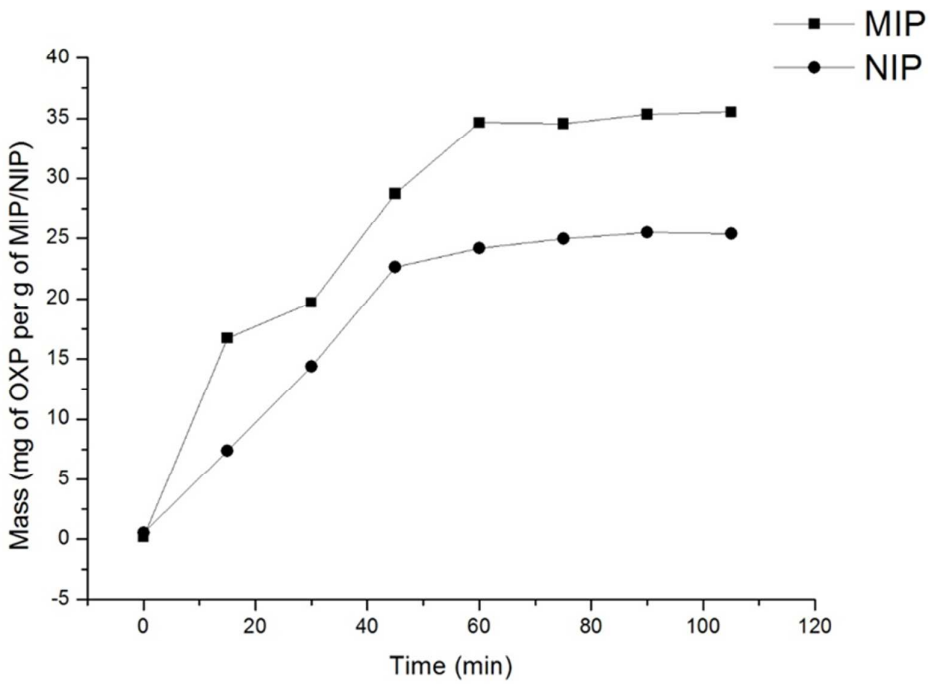


Figure 5

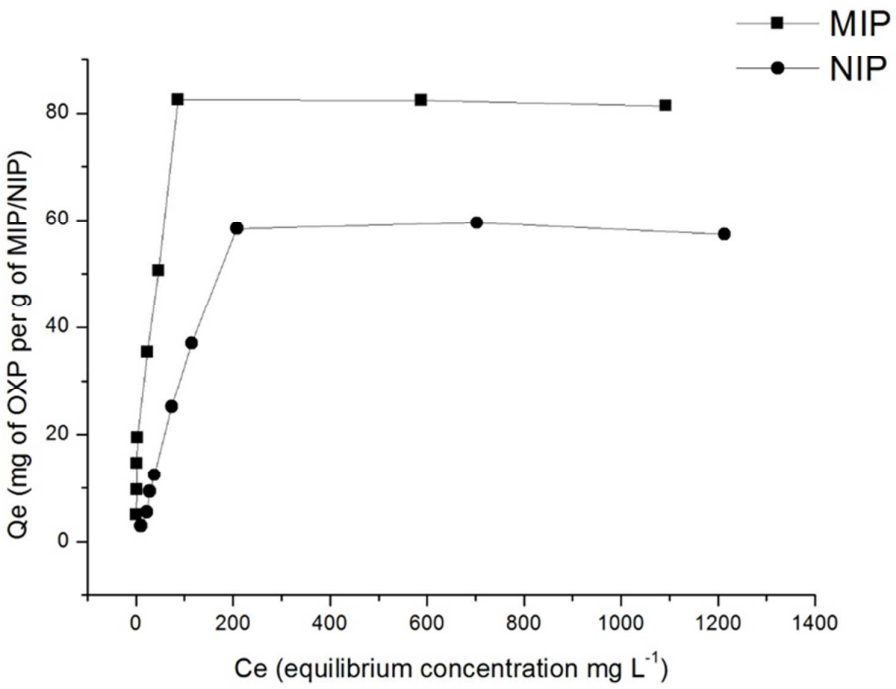


Figure 6

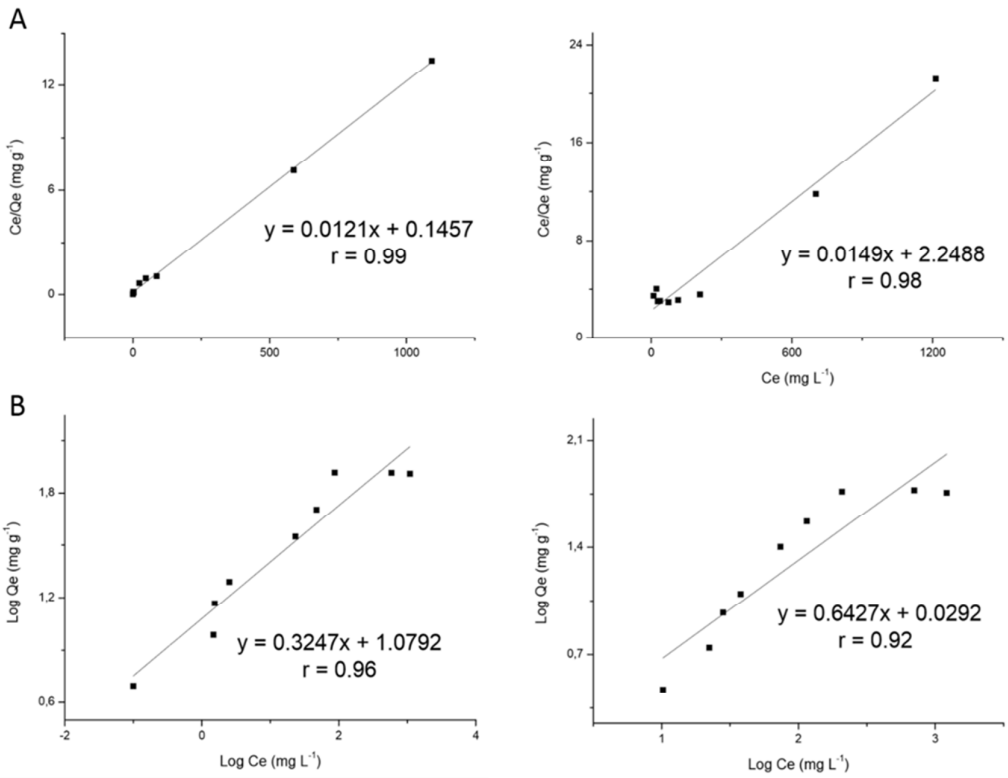
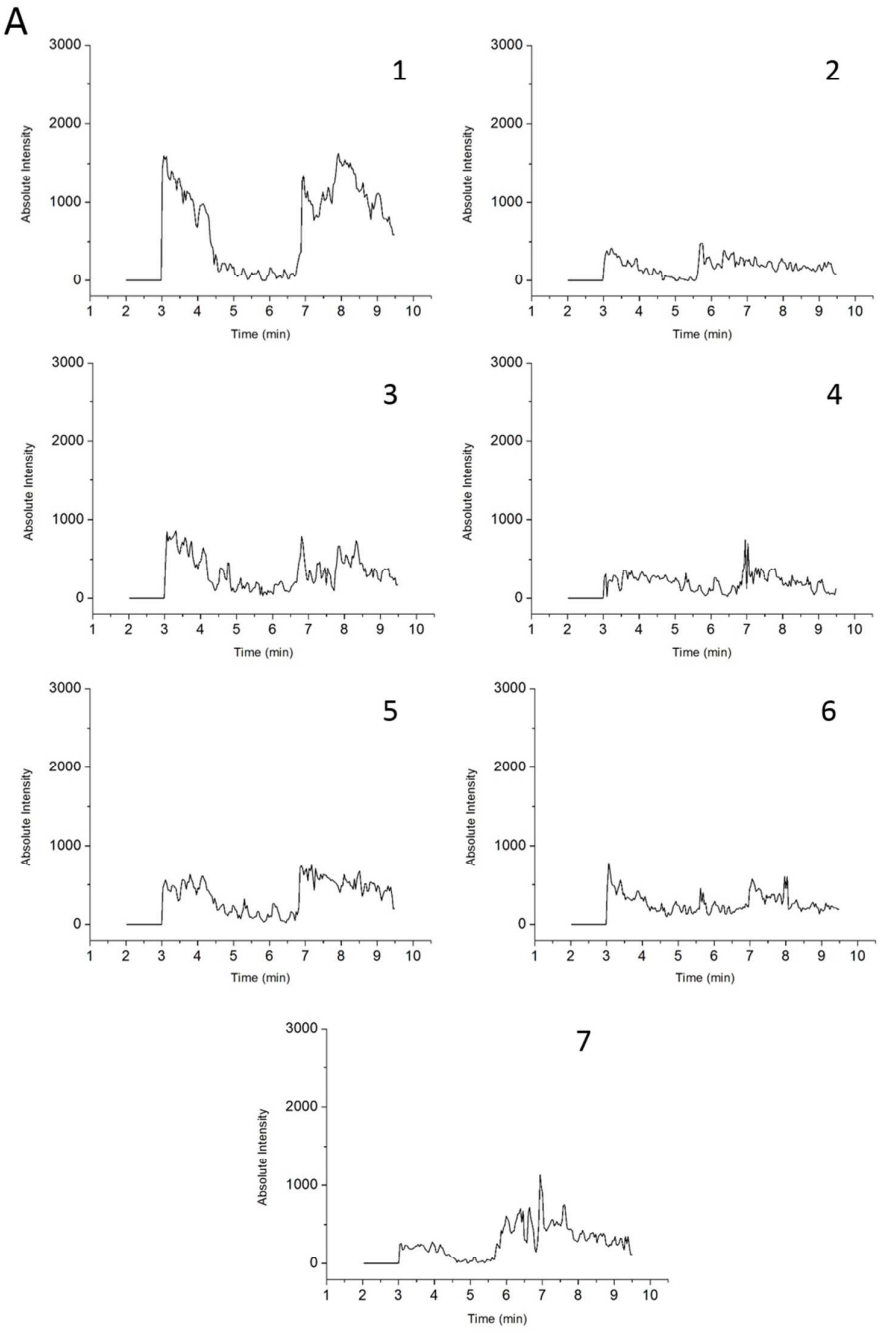
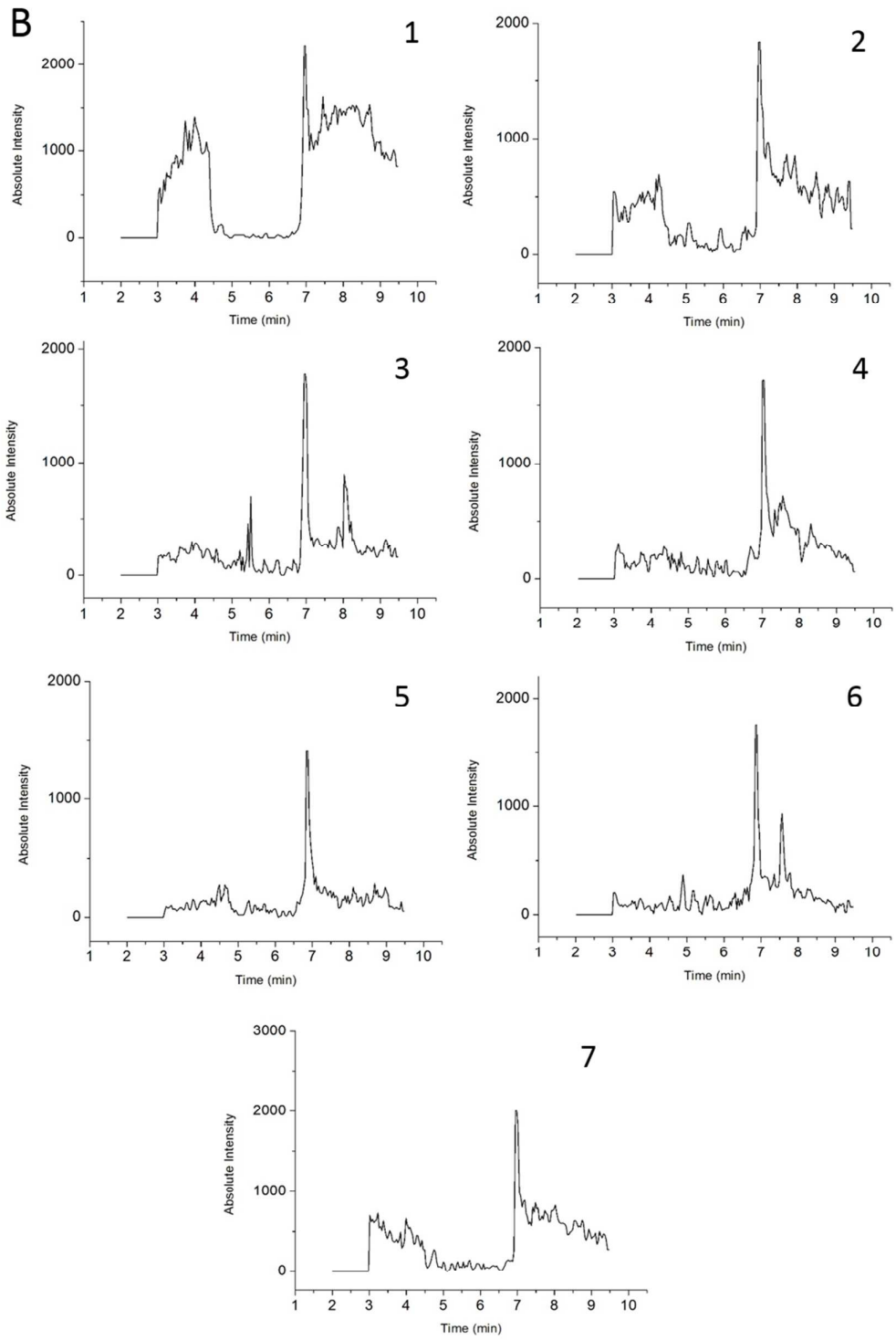
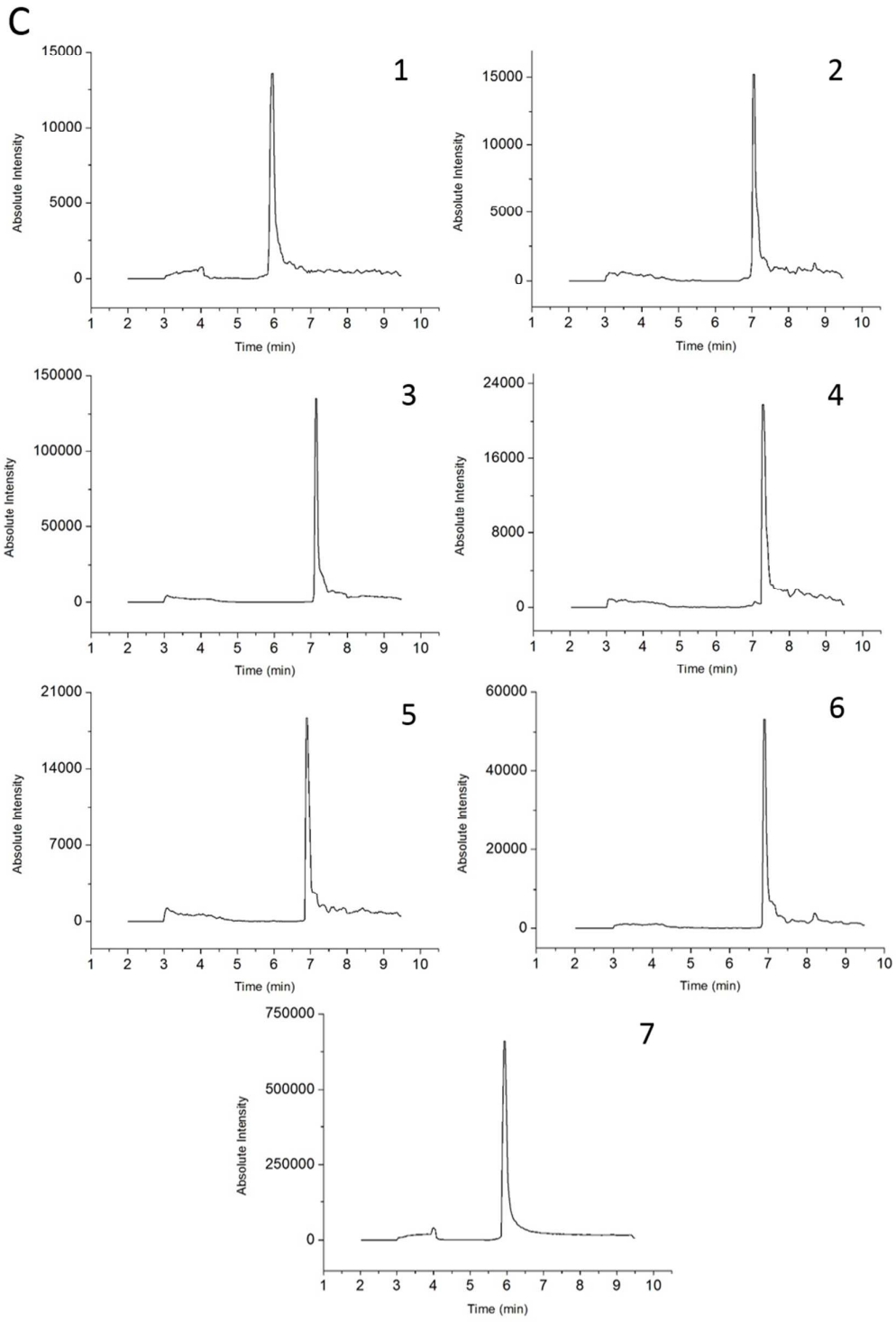
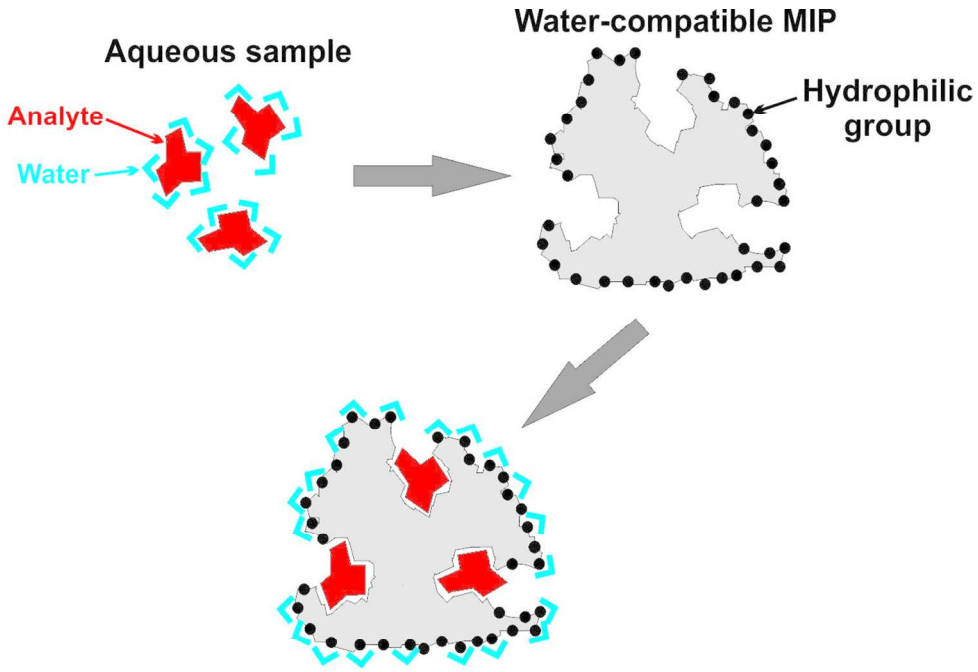


Figure 7









The hydrophilic layer forms hydrogen bonds with water, minimizing the interference of this solvent in the analyte-polymer complex.

115x84mm (300 x 300 DPI)