Solid-Phase Extraction and Determination of Metoclopramide in Biological Fluids and Human Urine by Molecularly Imprinted Polymers

N. Shaabani*
Young Researchers Club, Chemistry Department, Shahre-rey Branch, Islamic Azad University, Tehran, Iran.
M. Javanbakht
Department of Chemistry, Amirkabir University of Technology, Tehran, Iran.
L. Safaraliee, S. Eynollahi
Young Researchers Club, Center of Excellence in Electrochemistry, Tehran University, Tehran, Iran.

Abstract

Introduction: The imprinting technique involves the formation of a complex between a template molecule and the functional monomers in an appropriate solvent. The procedure for synthesizing an MIP is based on the chemical polymerization of a functional monomer and a cross-linking agent in the presence of a molecule used as a template. Removal of the template yields a functional polymeric matrix with recognition sites complementary in functionality and shape to the print molecule structure. These MIPs have been employed in fields where a certain degree of selectivity is required.

Aim: Simple and inexpensive extraction and detection of Metoclopramide (MCP) from biologic samples

Materials and Methods: After polymerization of SPE procedure, successful imprinting was confirmed by comparison of the recoveries between NIP and MIP particles. Molecular recognition properties, binding capability and selectivity of the MIPs were evaluated. UV spectroscopic determination of MCP was done at 309 nm and accuracy and precision was checked by the liquid chromatographic technique.

Results: Polymer imprinted for MCP has been synthesized via a non-covalent molecular imprinting approach. The MIP has then been applied in a SPE procedure, which enables the selective extraction of MCP from biological samples, even when MCP is present at low concentrations. It was shown that recoveries of nearly 99% of a MCP standard solution and up to approximately 91% from spiked human urine and serum samples could be obtained after SPE. The binding capacity of the MIP for MCP was determined to be 113 mg g-1 (319 μmol/ g). The MISPE can be successfully applied to clean-up and preconcentration of MCP.

Conclusion: Water-compatible molecularly imprinted polymers (MIPs) designed to enable the selective extraction of metoclopramide (MCP) from complex matrices such as biological fluids and human urine have been synthesized.

Keywords: Molecularly Imprinted Polymer, Solid-Phase Extraction, Metoclopramide, Biological Fluids, Human Urine

* Corresponding author

Introduction

The molecular imprinting technique has emerged as a powerful approach for the creation of recognition sites in highly cross-linked polymeric matrices .[1] The imprinting methodology involves copolymerization of methacrylic acid (MAA) monomers with ethylenglycol-dimethacrylate monomers in the presence of guest molecules to produce three-dimensional network polymers. Removal of the template yields a functional polymeric matrix with recognition sites complementary in functionality and shape to the print molecule structure. These imprints exhibit selectivity for rebinding the template with which it was prepared. These MIPs have been employed in fields where a certain degree of selectivity is required such as sensors [2], chromatography [3], and catalysis. [4] Solid-phase extraction (SPE) has become a routine tool in many laboratories for preconcentration and clean-up steps in the analysis of complex samples. Due to the popularity of SPE, new solid sorbents have appeared as alternative to conventional sorbents with the aim of achieving a more and more selective preconcentration of target analytes. Thus, immunosorbents and synthetic receptors such as molecularly imprinted polymers (MIPs) appear as excellent candidates to accomplish this requirement. [5] The materials routinely used in SPE are usually based on the non-specific binding of the targets. Molecularly imprinted polymers (MIPs) offer the possibility of achieving selective extraction, analogous to those achieved by immuno based extraction systems, and thus may represent an advance on conventional SPE materials. [6] The application of MIP technology to solid-phase extraction (MISPE) has been used for both biological and environmental samples and has been reviewed. [7-9] In spite of their attractive features, the classical SPE sorbents retain analytes by nonselective hydrophobic or polar interactions that lead to a partial coextraction of interfering substances. In order to enhance the selectivity of the extraction, the MIPs were recently developed.

Newly, we described the use of MIPs as new sensing material in potentiometric detection of hydroxyzine [10] and cetirizine. [11] In this work, we present a new sorbant based on MIPs for selective solid phase extraction of MCP and its spectrophotometric determination at 309 nm. This scheme allows the sensitive, simple, and inexpensive extraction and detection of the analyte without using additional reagents or instruments, and accuracy checked with the HPLC method.

Metoclopramide(MCP),4-amino-5-chloro-2-methoxy-N-(2-diethylamino-ethyl), benzamide is a dopamine-receptor antagonist active on gastrointestinal motility. It is used as an anti-emetic in the treatment of some forms of nausea and vomiting to increase gastrointestinal motility. It is also used at much higher doses for the prevention of cancer chemotherapy-induced emesis.[12] Both the British Pharmacopoeia (BP)[13] and the United States Pharmacopoeia (USP)[14] recommend a nonaqueous acid–base titration with potentiometric detection of the end-point for the evaluation of the raw material of metoclopramide; for its dosage forms, the BP describes spectrophotometric methods while the USP recommends HPLC methods. Most of the analytical methods employed for the determination of metoclopramide in dosage forms or in biological fluids are chromatographic methods including HPLC,[15] LC,[16] reversed-phase HPLC,[17] HPTLC,[18] GC–MS,[19] electron-capture GC,[20] and high-performance capillary electrophoresis (HPCE).[21] Also, spectrophotometric methods are among the most analytical methods used for metoclopramide determination in its dosage forms, in biological fluids or in mixtures with other drugs.[22-27] Other reported methods include titrimetry,[28] differential scanning calorimetric (DSC) and X-ray diffraction,[29] colorimetric,[30] fluorimetry,[31] fast stripping continuous cyclic voltammetry,[32] voltammetry,[33] 1H-NMR spectroscopy,[34] flameless atomic absorption spectrophotometry[35] and radio-immunoassay. [36]

Matherian and Method

Methacrylic acid (MAA) from Merck (Darmstadt, Germany) was distilled in vacuum prior to use in order to remove the stabilizers. Ethylene glycol dimethacrylate (EGDMA) and 2, 2'-azobis isobutyronitrile (AIBN) from Merck (Darmstadt, Germany), were of reagent grade and were used without any further purification. The phosphate buffer solutions with a pH value of 10.0 were prepared in de-ionized water and were used. All the other chemicals were of analytical reagent grade and the solutions were prepared with distillated water. Drug free human serum was obtained from the Iranian blood transfusion service (Tehran, Iran) and stored at -20°C until use after gentle thawing. Urine was also collected from healthy volunteers.

MIP and NIP preparation with precipitation polymerization

The schematic representation of the imprinting and the removal of MCP from the imprinted polymer are shown in Fig. 1. The monomer MAA (174 µL, 2.04 mmol), MCP print molecule (77 mg, 0.217 mmol) and 30 mL of chloroform were placed in a glass sample vial. Then cross-linker EGDMA (1.87 mL, 9.90mmol) was added. The mixture was uniformly dispersed by sonication (sonic bath model Ultrasonic UTD35-Falc, Via Piemonte, Italy). After sonication, it was purged with N2 for 10 min and the glass tube was sealed under this atmosphere. Then the reaction initiator AIBN (57 mg, 0.347 mmol) was added. The polymerization was carried out for 24 h in a water bath 60 °C. After the polymerization procedure and drying, the polymer particles were washed with methanol and acetic-acid (10:1, v/v, of 98 % methanol and pure acetic acid) for three times and with distillated water for two times. The complete removal of template was followed by spectrophotometer of UV, after three times washing it was not seen spectra of MCP. In order to verify that retention of template was due to molecular recognition and not to non-specific binding, a control (non-imprinted polymer) was prepared following the same procedure, including washing, but with the omission of the target molecule, MCP.

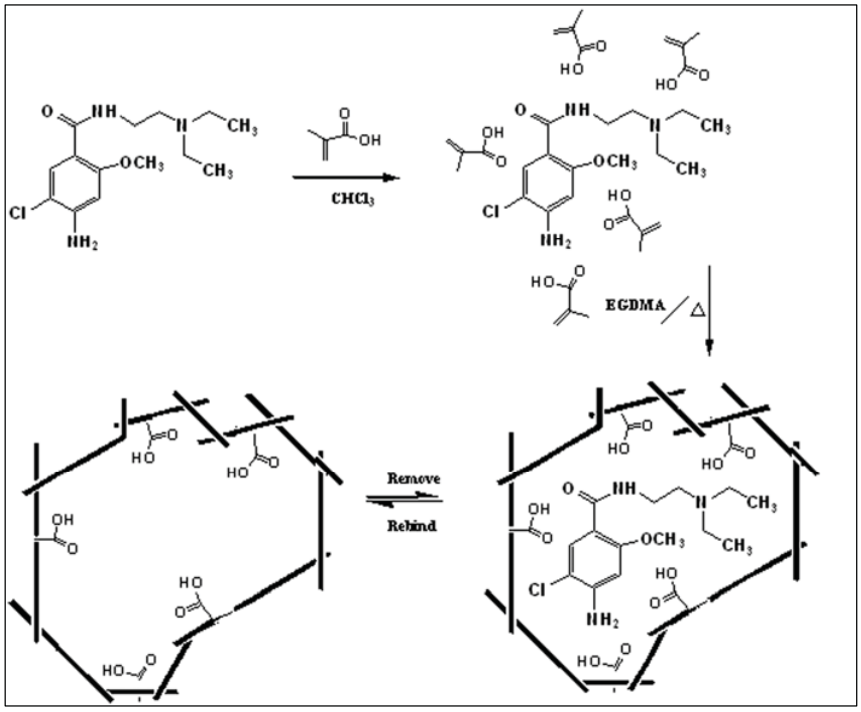

Fig. 1 - Schematic representation of the MIP synthesis

Instrumentation

Apparatus

pH of solutions were adjusted using a model 630 digital Metrohm pH meter equipped with a combined glass–calomel electrode. The thermal analysis of polymer was carried out on a model PL-STA-1500 thrmogravimetric analysis (TGA)–differential thermal analysis (DTA) instrument from Polymer Labrotories' Company (Church Stretton, Shropshire, UK). 5.00 mg of the grounded polymer was heated at a heating rate of 5 ˚C /min from ambient temperature up to 600 ˚C under nitrogen atmosphere (flow rate = 20 mL/ min) and the corresponding TG curves were obtained.

Thermo gravimetric Analysis

Fig. 2 depicts the TGA plots of the unleached and leached MIP particles. Regarding the unleached MIP particles, TGA revealed two decomposition states: one mass loss starting at 78.7 ˚C downward, assigned to the decomposition of the free monomer and the cross-linker, and one starting at ~239˚C, related to the MCP hydrochloride decomposition as the melting point of MCP hydrochloride is 183 ˚C. [37] All the materials were completely decomposed prior to reaching the temperature of 469 ˚C. These observations indicated the rigidity of the unleached and leached MIP particles is further than blank materials, as the formers exhibits decomposition above ~370 ˚C, the latter starts its decomposition at ~250 ˚C onwards.

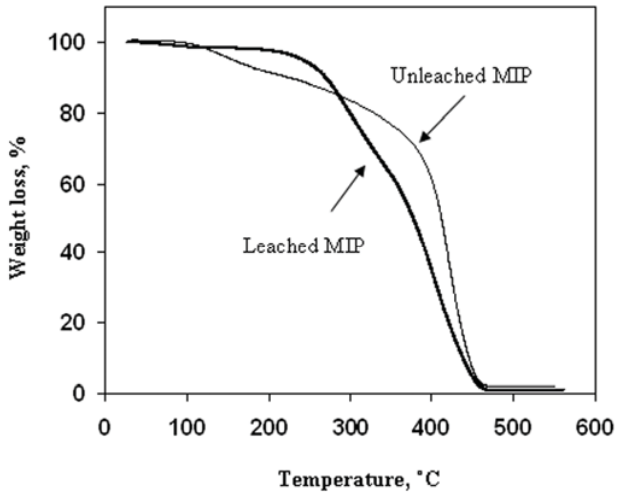

Fig. 2- TGA plots of the unleached and leached MIP particles

Procedures

Batch Binding Assay

Adsorption of drug from aqueous solutions was investigated in batch experiments. The general procedure for extraction of MCP by the MIP was as follows: The polymer beads were suspended in aqueous solutions and the pH was adjusted at 10.0. In all experiments, the polymer concentration was kept constant at 100 mg/50 mL. The mixtures were thermo stated at 25 °C for 20 min. under continuous stirring and then was filtrated on a paper filter (flow rate= 100 ml/ min by applied vacuum). The concentration of free MCP in the filtrate was determined by spectrophotometer of UV. The instrument response was periodically checked with known MCP standard solutions. Three replicate extractions and measurements were performed for each aqueous solution. Percent extraction of MCP was calculated from the following equation:

$$\text{Extraction\%} = \frac{C_i - C_f}{C_i} \times 100 \quad (1)$$

Where C_i and C_f are the concentrations of MCP before and after extraction in the solution. The adsorbed MCP was desorbed from the MIP by treatment with 5 mL of methanol and acetic acid (10:1, v/v, of 98% methanol and pure acetic acid). The imprinted polymer containing MCP was placed in the desorption medium and stirred continuously at 600 rpm and room temperature for designated time. The final MCP concentration in the aqueous phase was determined by spectrophotometer of UV. The same procedure was followed for NIP particles.

Table 1- Compositions and comparisons of the extraction of MCP from MCP standard solution (0.1 mM) using polymers as sorbents

MIP	MAA (mmol)	VPM (mmol)	EGDMA (mmol)	AIBN (mmol)	Extraction(%) (mean ± SD)[a]
MIP1	0.90	0.217	9.90	0.347	50.5 (± 3.6)
MIP2	2.04	0.217	9.90	0.347	98.5 (± 4.1)
MIP3	5.04	0.217	9.90	0.347	30.2 (± 2.7)
MIP4	7.57	0.217	9.90	0.347	20.9 (± 3.5)
MIP5	2.04	0.217	6.88	0.347	28.0 (± 2.8)
MIP6	2.04	0.217	20.6	0.347	50.3 (± 3.1)
MIP7	2.04	0.217	27.5	0.347	60.6 (± 4.5)
NIP1	0.9	----	9.90	0.347	10.7 (± 1.2)
NIP2	2.04	----	9.90	0.347	27.0 (± 3.4)
NIP3	5.04	----	9.90	0.347	18.5 (± 1.8)
NIP4	7.57	----	9.90	0.347	24.0 (± 2.6)

[a]Average of five determinations

Extraction procedure for plasma and urine samples

Drug free human serum was obtained from the Iranian blood transfusion service (Tehran, Iran) and stored at -20 °C until use after gentle thawing. Urine was also collected from healthy volunteers (males, around 35-years-old). The samples were centrifuged for 20 min at 8000 rpm and then filtered through a cellulose acetate filter (0.20 µm pore size, Advantec MFS Inc. CA, USA). The filtrate were collected in glass containers and stored at -20 °C until analysis was performed, with the minimum possible delay. Stock standard solutions of MCP were prepared in water. Working standard solutions were prepared by adding appropriate volumes of MCP solution to a 5 mL volumetric flask and the solution was diluted to the mark with biological fluids and vortexes for 10 minute. Then the solution was adjusted to pH=10.0 and the analysis was followed up as indicated in the general analytical procedure.

Results and discussion

There are several variables, such as amount of monomer or nature of cross-linker and solvent that affects the final characteristics of the obtained materials in terms of capacity, affinity, and selectivity for the target analyte. Primary experiments revealed that the imprinted polymers prepared in chloroform show better molecular recognition ability in aqueous environment than MIPs prepared in acetonitrile. Thus, in chloroform, different formulations for the obtainment of MIPs with improved molecular recognition capabilities have been used (Table 1). Generally, proper molar ratios of functional monomer to template are very important to enhance specific affinity of polymers and number of MIPs recognition sites. High ratios of functional monomer to template result in high non-specific affinity, while low ratios produce

fewer complexation due to insufficient functional groups. [38] Five molar ratios of the monomer MAA to the template of 4:1, 9:1, 23:1, and 34:1 were used in the experiments. The optimum ratio of functional monomer to template for the specific rebinding of MCP was 9:1 (Table 1), which had the best specific affinity and the highest recovery of 95.8%, while that of the corresponding NIPs was low at 25%. The specific adsorption recovery of MCP at 9:1 was 25%, while those at 4:1, 23:1 and 34:1 were 10.7%, 18.5% and 24%, respectively. For the polymers with a ratio of 23:1 and 34:1, an excess of the functional monomer with respect to the template yielded higher non-specific affinity. Therefore, the typical 1:9:45 template:monomer:cross-linker molar ratio was used for further studies.

Effect of pH

The effect of pH on the sorption of MCP was investigated by varying the solution pH from 1.5 to 10.0. Several batch experiments were performed by equilibrating 50 mg of the imprinted particles with 25 mL of solutions containing 0.1 mM of MCP under the desired range of pH. The pH dependence of extracted percentage of MCP is shown in Fig. 3. As seen, binding of MCP increased with increasing pH and reached to maximum at pH of 8.5–10.0. At low pHs, the nitrogen hetero-atoms can be protonated and, therefore, negligible amounts of MCP are adsorbed to the polymer.

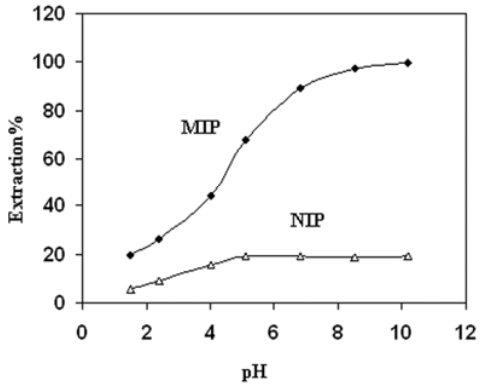

Fig. 3- Effect of pH on sorption of MCP on imprinted polymer particles.

The Effect of the Extraction Time

The effect of the extraction time on the efficiency of the extraction for a seies of 50 mL of 0.1 mM MCP solutions was investigated. As it is seen, the time of the extraction from 1 to 60 min. The best time for extraction was after 15min.

Choice of washing and eluent solution

Optimization of the washing procedure is critical in MISPE. The selectivity of MISPE is generally obtained by the introduction of a selective washing procedure in order to remove compounds retained only by non-specific interactions. A MCP solution (50 mL of 0.10 mM) was loaded on MIP and NIP. For the washing step, 3 mL water followed 2 mL acetone eliminated MCP from NIP by suppression of non-specific interactions (Table 2). These washing solutions are able to disrupt Van der Waals interactions and, probably, a part of the hydrogen bonds thanks to theirs hydrogen bond donor properties. Finally, in order to choose the most effective eluent for desorbing MCP from the sorbent, a series of selected eluent solution such as methanol, ethanol and acetonitril (10:1, v/v, of eluent and pure acetic acid) a total of 5.0 mL of the above mentioned eluents were used for desorbing the adsorbed MCP. The results showed that recovery was the best (95.5 %) when mixed solution of methanol and acetic acid (10:1, v/v) was used as eluent (Table 2). Indeed, MeOH and CH3COOH are protic

and polar solvents able to break hydrogen bonds between functional groups of MCP and carboxyl groups present in MIP cavities.

Table 2- Recoveries (%) obtained from after the loading of 100 mg of MIP and NIP with 5 µmol of MCP

No.	Fraction	Recovery %	
		MIP	NIP
1	Washing, 3 mL, water	Not detected	5 ± 1.1
2	Washing, 2 mL, acetone	3.5 ±1.0	12 ± 1.4
3	Elute, 5 mL, methanol and pure acetic acid (10:1, v/v)	95.5 ± 3.3	10 ± 1.3
4	Ethanol and pure acetic acid (10:1, v/v)	76.5 ± 3.0	12 ± 1.6
5	Acetonitril and pure acetic acid (10:1, v/v)	53.0 ± 2.5	9.5 ± 1.2
	Total (Nos. 1, 2 and 3)	99.0 ± 3.4	27.0 ± 2.2

[a] Average of three determinations

Effect of amount of MIP

In order to investigate the optimum amount of MIP on the quantitative extraction of MCP, the extraction was conducted by varying the amount of the MIP from 50 to 200 mg. The extraction of MCP is quantitative by using 100 mg of MIP.

Adsorption capacity

The capacity of the sorbent is an important factor that determines how much sorbent is required to remove a specific amount of drug from the solution quantitatively. For investigation of adsorption of MCP, the same volumes of MCP solution (50 mL) with different concentrations of MCP were contacted with 100 mg of sorbent in the batch mode. Then, the concentration of the remaining MCP in the solution was determined by UV. The adsorption isotherm that is the number of milligram absorbed per gram of adsorbent (N) versus the equilibrium concentration of MCP is shown in Fig. 4. According to these results, the maximum amount of MCP that can absorb by MIP was found to be 196 mg/ g (319 µmol/ g) at pH 10.0. Taking into account template-monomer ratio during MIP preparation, the theoretical number of imprints was 1.4 mmol per gram of polymer. However, experimental data show strong specific retention of MCP (319 µmol per gram of MIP), which means that approximately thirty percent of the theoretical number of sites was formed. Such behavior is coherent with previous data [39, 40] with MIP capacities ranged from 10 to 1000 µmol per gram. For higher MCP amounts (higher than 319 µmol/ g), a slight increase of retained MCP was observed on MIP capacity curve. As all the accessible specific cavities of the MIP are saturated, the retention of the analyte is only due to non-specific interactions, which can be identical for MIP and NIP polymers.

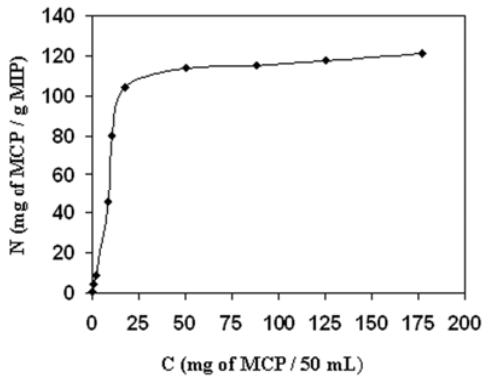

Fig. 4- Curve of capacity obtained after the loading of 5 mL aqueous solution spiked with increasing amounts of MCP onto the MIP particles.

Calibration curve and precision

Under the optimized conditions, calibration curves were obtained by the MISPE protocol measured at ten increasing concentrations, in a range from 1 to 200 μM of MCP. The results showed good linearity (r=0.9957) in the dynamic range of 50.0–1000 μM. The results confirmed that the SPE method based on MIP beads could be directly applied to real sample analysis.

According to the signal-to-noise relation rule equal to 3.0, the limit of detection for MCP was 3 μM. The precision of the method were assessed by performing replicate analyses of quality control samples at three different concentrations of MCP (10, 50 and 100 μM) in four replicates in the same day and consecutive days. The results showed that the intra and inter-assay relative standard deviations and inter-assay relative standard deviations of the proposed method were lower than 5.4% and 5.2%.

Study of MIP selectivity

The MIPs are usually evaluated to check theirs recognition properties for a target analyte. Chromatographic evaluation and equilibrium batch rebinding experiments are the methods most commonly used to investigate the selectivity of the imprinted materials. [41, 42] For equilibrium batch rebinding experiments, a known mass of template in solution is added to a vial containing a fixed mass of polymer. Once the system has come to equilibrium, the concentration of free template in solution is measured and the mass of template adsorbed to the MIP calculated. [43- 44] In order to measure the selectivity of the imprinted material, the sorptions were investigated in batch experiments. The initial concentrations of drugs (50 μg/ mL) were extracted by 100 mg of imprinted material at a pH of 10.0 on MIP and NIP. As can be seen in Table 3, distribution ratio (KD) and selectivity coefficient of the sorbent (ksel) were obtained in these competitive experiments. The distribution ratio (mL/ g) of MCP between the MIP particles and aqueous solution was determined by following equation:

$$K_D = \frac{(C_i - C_f)V}{C_f m} \quad (2)$$

Where V is the volume of initial solution and m is the mass of MIP materials. Selectivity coefficients for MCP ion relative to foreign compounds are defined as:

$$k^{sel}_{VPM/j} = \frac{K_D^{VPM}}{K_D^{j}} \quad (3)$$

Where K_D^{VR} and K_D^{j} are the distribution ratios of MCP and foreign compound, respectively. The previous MISPE protocol was applied also to these drug molecules on NIP particles. The relative selectivity coefficient (k') was also determined by following equation:

$$k' = \frac{k(MIP)}{k(NIP)} \quad (4)$$

In the case of MCP, a quantitative extraction and an excellent MIP/NIP selectivity were obtained.

Table 3- Distribution ratio (KD), selectivity coefficient (k^{sel}) and relative selectively coefficient (k') values of MIP and NIP material for different drugs.

Drug	K_D(MIP) (mL g^{-1})	K_D(NIP) (mL g^{-1})	k^{sel}(MIP)	k^{sel}(NIP)	k'
Metoclopramide	9500	55.5	---	---	---
Terazosine	214.2	45.1	44.3	1.23	36.0
Hydroxizine	159.6	88.2	60.5	0.62	97.5
Verapamil	125.7	86.1	75.5	0.64	118.0
Felurazepam	91.7	42.8	103.5	1.3	75.3
Imipramine	63.0	29.1	150.7	1.91	79.0

Metoclopramide assay in spiked human serum and urine

The extraction procedure has to be optimized in order to eliminate low energy interactions at the surface without damaging specific interactions taking place in the cavities and that are of stronger energy due to the spatial recognition. The procedure of extraction has to be tested in parallel with the NIP, to evaluate the risk of non-specific interactions with the external surface of the MIP. Therefore, the procedure should be based on the use of a solvent for the percolation step and/or the washing step that possess an elution strength sufficiently high to disrupt the interactions that can take place with residual monomers at the surface of the polymer without affecting the overall retention in the imprints. Thus, the proposed SPE procedure was successfully applied to assay of MCP in spiked human serum and urine. [45] The obtained results (Table 4) were statistically compared with those obtained by the official chromatographic method. [46] Since the calculated value of F does not exceed the theoretical value (Table 4), there was no significant difference between the described and reported methods with respect to reproducibility.[47] Also, no significant difference was noticed between the two methods regarding accuracy and precision as revealed by t-value [47], Table 4. This means that the proposed procedure should be applicable to the analysis of this and other similar fluids containing MCP.

Table 4- Assay of MCP in Human serum and urine by means of the described MISPE procedure and the official chromatographic method

Sample	Spiked Value (µM)	Proposed SPE Procedure (Found ±SD) (µM)		HPLC
		MIP	NIP	
Human Serum	10	9.1 ± 0.5	1.0 ± 0.2	8.9 ± 0.4
	50	43.5 ± 2.2	4.3 ± 0.4	-
	100	83.3 ± 4.7	9.5 ± 0.8	85.5 ± 4.8
Human Urine	10	9.1 ± 0.6	1.5 ± 0.5	9.0 ± 0.4
	50	45.1 ± 2.2	4.8 ± 1.1	47.6 ± 2.5
	100	89.4 ± 5.1	11.3 ± 2.4	-

Conclusions

It will be necessary to develop new MIP synthesis methods to improve the capacity and/or selectivity of MISPE sorbents. In this paper, a polymer imprinted for MCP has been synthesized via a non-covalent molecular imprinting approach. The MIP has been applied in a SPE procedure, which enables the selective extraction of MCP from biological samples, even when MCP is present at low concentrations. It was shown that recoveries of nearly 99% of a MCP standard solution and up to approximately 91% from spiked human urine and serum samples could be obtained after SPE. The binding capacity of the MIP for MCP was determined to be 113 mg/g (319µmol/g). The MISPE can be successfully and with a high efficiency applied to clean-up and preconcentration of MCP among simple procedures, and simultaneously it can greatly increase the sensitivity of conventional chromatographic methods.

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