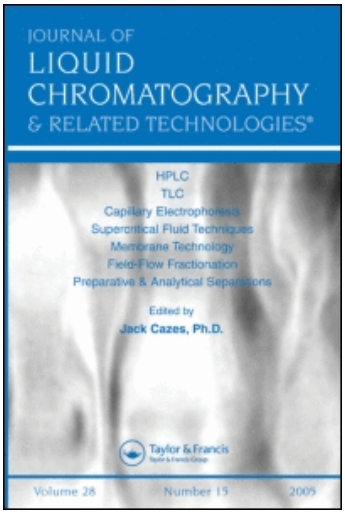

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M. Lachová[a]; J. Lehotay[a]; I. Skačáni[a]; J. Čižmárik[b]
[a] Institute of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava, Slovak Republic [b] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Comenius University in Bratislava, Slovak Republic

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Isolation of Some Derivatives of Phenylcarbamic Acid from Human Plasma using Molecularly Imprinted Polymers

M. Lachová,[1] J. Lehotay,[1] I. Skačáni,[1] and J. Čižmárik[2]

[1]Institute of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava, Slovak Republic
[2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Comenius University in Bratislava, Slovak Republic

Abstract: Two molecularly imprinted polymers for 1-methyl-2-piperidinoethyl ester of 4-decyloxyphenylcarbamic acid (template) have been synthesized using a non-covalent imprinting approach. Acrylamid and methacrylic acid, respectively, were used as the functional monomers. Imprinted polymers were used as the sorbents for solid phase extraction (MISPE). The capacity and selectivity of prepared imprinted polymers was investigated. The non-imprinted (blank) polymers were prepared by the same way, without the template, to study the non-specific interactions. Molecularly imprinted polymers were used to extract derivatives of alkoxyphenylcarbamic acid from spiked human plasma. C_{18} sorbent was also used for SPE to compare the efficiency of the procedure.

Keywords: HPLC, Molecularly imprinted polymer, SPE

INTRODUCTION

Solid phase extraction (SPE) is the most popular sample pretreatment method today. Presently, there are several types of sorbents for SPE

Correspondence: J. Lehotay, Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava, Radlinského 9, 812 37 Bratislava, Slovak Republic. E-mail: jozef.lehotay@stuba.sk

including normal phase, reversed phase, ionic, and other special sorbents. However, due to their unsatisfactory selectivity, these traditional sorbents usually can not separate analytes efficiently in complex biological or environmental samples.[1]

Molecularly imprinted polymers (MIPs) are tailor-made materials with high selectivity for a target molecule. This selectivity arises from the synthetic procedure followed to prepare the MIP, in which a template molecule is linked, by covalent bonds or non-covalent forces, to suitable monomer(s) containing functional groups. This link is responsible for the subsequent specific binding sites imparted to the MIP.[2]

MIPs are stable towards a wide range of solvents, are highly thermostable, can be used over a range of temperatures.[3] They can be stored at ambient temperature and in dry state without loss of performance.

In general, molecular imprinting can be achieved in three ways: The non-covalent approach is based on the formation of a prepolymerization complex between monomers carrying suitable functional groups and the template, through non-covalent bonds such as ionic interactions or hydrogen bonding. The template can be removed after the polymerization simply by solvent extraction. The covalent imprinting, polymerizable derivatives of the template molecule are copolymerized with a crosslinking monomer. These derivatives are obtained by forming covalent bonds between the template and suitable polymerizable monomers. To remove the template from the polymer, these covalent bonds have to be chemically cleaved.[4] Semi-covalent approach is a hybrid of the two previous methods. Covalent bonds are established between the template and the functional monomer before polymerization, once the template has been removed from the polymer matrix, the subsequent rebinding of the analyte to the MIP exploits non-covalent interactions, as per the non covalent protocol.[5]

MISPE can be used to pre-enrich the target analyte and also to remove other compounds from the sample matrix. In recent years, MISPE has been added to the extraction of several compounds from different matrices, such as environmental samples (river water, ground water, wastewater, sea water, and soil extracts),[6–9] biofluids (urine, serum, plasma, blood),[10–12] tissue samples,[13,14] food samples,[15,16] and plants.[12,16,17]

The aim of our work is to estimate the influence of length and position of the alkoxygroup in the 1-methyl-2-piperidinoethyl ester of alkoxyphenylcarbamic acid derivates on the binding capacity of MIP on the base polymethacrylic acid and polyacrylamide. 1-methyl-2-piperidinoethyl ester of 4-decyloxyphenylcarbamic acid was used as the template. The capacity of MIP was compared with the non imprinted polymers prepared in the same way without the template in the polymerization mixture. The prepared MIP was also used for SPE

of 1-methyl-2-piperidinoethyl ester of alkoxyphenylcarbamic acid derivates from spiked human plasma.

EXPERIMENTAL

Materials

1-Methyl-2-piperidinoethyl esters of alkoxyphenylcarbamic acids were synthesized by the Pharmaceutical Faculty.[18] Acetonitrile, methanol, toluene, methacrylic acid, and diethylamine were purchased from Merck, acrylamide, azobisizobutylonithrile (AIBN), and ethyleneglycoldimethacrylate (EGDMA) were obtained from Fluka, and acetic acid was purchased from Lachema. SPE cartridges Sep-Pak 3cc C18 were from Waters.

HPLC Analysis

An HP 1100 system (Hewlett-Packard, Germany), consisting of a pump with a degasser, a diode-array detector (DAD), a 50 μL injector, and a HP ChemStation were used. Analyses were carried out in the analytical column Separon SGX C18 (125 × 4 mm, 7 μm) (Watrex, USA) at laboratory temperature. Mobile phase consisted of methanol, acetonitrile, acetic acid, and diethylamine (80:20:0.1:0.1), at a flow rate of 0.5 mL/min. Izokratic elution was used. Diode-array detector worked in the range of 190–400 nm and the chromatograms were acquired at wavelength of 240 nm.

Polymer Preparation

The molecularly imprinted polymers were prepared according to Zhang et al.'s method.[19] Two MIPs were synthesized, using acrylamide (MIP1) and methacrylic acid (MIP2), respectively, as a functional monomer. The monomer (1.8 mmol), the template molecule 1-methyl-2-piperidinoethyl ester of 4-decyloxyphenylcarbamic acid (0.3 mmol), and the porogen acetonitrile (3 mL) were placed into a glass tube. Then the crosslinker EDMA (9 mmol) and the initiator AIBN (9 mg) were added. The polymerization was carried out in a water bath at 60°C for 24 h. The prepared polymer was passed through a 40 μm sieve, fine particles were removed by flotation in acetone, and the final product was dried under vacuum at 60°C for 1 h. The template was removed from the MIP by Soxhlet extraction with 70 mL of a mixture of methanol/acetic acid (9:1, v/v) until it was not detected in the extract. Non-imprinted, control polymers

(NIPs) were prepared in the same manner as MIPs but in the absence of the template molecule in the polymerization mixture.

Evaluation of MIP

Each polymer of 100 mg was packed in 3 mL polypropylene cartridges. The cartridges capacity was tested in methanol, acetonitrile, and water. Prior to applying the solution of ester of 4-decyloxyphenylcarbamic acid (4-DPCA), the polymer was pre-equilibrated with 5 mL of methanol and then with 5 mL of solvent in which the capacity was studied. 4-DPCA solution (0.5 μg/mL for MIP1, NIP1, and 2 μg/mL for MIP2, NIP2) was added into the cartridge until release was detected. All effluents were measured by HPLC.

Selectivity of MIPs for ester of 4-methoxyphenylcarbamic acid (4-MPCA) and of 2-decyloxyphenylcarbamic acid (2-DPCA) was tested. Selectivity of MIP1 in acetonitrile and of MIP2 in methanol was studied by the same procedure as described for the template. The concentration of 4-MPCA and 2-DPCA was 0.5 μg/mL for MIP1, NIP1, and 2 μg/mL for MIP2, NIP2.

Then, washing solvents were tested. The 100 mg amount of each dried polymer was packed into the polypropylene cartridge. The cartridges were conditioned with 5 mL of solvent, which was used in the washing step and 5 mL of methanol (MIP1 and NIP1 also with 5 mL of acetonitrile). After the conditioning, the solution of 4-DPCA was added into a polymer catridge. 2 mL of analyte solution (0.5 μg/mL in acetonitrile) was added to MIP1 and 0.5 mL of solution (0.5 μg/mL in acetonitrile) was added to NIP1. The analyte solution of 4 mL (2.0 μg/mL in methanol) was added to MIP2 and 2 mL of analyte solution (2.0 μg/mL in methanol) was added to NIP2. Methanol, acetonitrile, water, mixture of methanol/acetic acid (99:1, v/v), mixture of methanol/acetic acid (95:5, v/v), and mobile phase were used as washing solvent. Each effluent was collected in 1 mL fractions. In the case of mixtures of methanol/aceacetic acid, all fractions were evaporated to dryness and redissolved in methanol. All fractions were analysed by HPLC.

The same washing solvents were tested for 4-MPCA and 2-DPCA. Amounts of 2-DPCA used in experiment: 2 mL (0.5 μg/mL in acetonitrile) to MIP1, 0.5 mL (0.5 μg/mL in acetonitrile) to NIP1, 2 mL (2.0 μg/mL in methanol) to MIP2, and 2 mL (2.0 μg/mL in methanol) to NIP2. Amounts of 4-MPCA used in experiment: 1 mL (0.5 μg/mL in acetonitrile) to MIP1, 0.5 mL (0.5 μg/mL in acetonitrile) to NIP1, 4 mL (2.0 μg/mL in methanol) to MIP2, and 2 mL (2.0 μg/mL in methanol) to NIP2. The amounts of analytes used for each polymer result from determined capacities of each polymer for all tested analytes.

MISPE Procedure

Polymers MIP1 and MIP2 (100 mg), respectively, were packed into polypropylene cartridges. SPE was performed on both MIPs and on the commonly used sorbent C_{18}. Cartridges were gradually conditioned with 5 mL of methanol, 5 mL of acetonitrile, and 5 mL of water. Then, 0.5 mL of spiked human plasma was added into each cartridge. The concentration of studied analytes (4-MPCA, 2-DPCA, and 4-DPCA) in plasma was 1 μg/mL for MIP1 and 10 μg/mL for MIP2 and sorbent C_{18}. Then the cartridges were washed with 1 mL of water and dried. Then dry MIPs were washed with 1 mL of acetonitrile (MIP1) or methanol (MIP2) and dried again. Analytes were eluted by 1.5 mL of a mixture of methanol/acetic acid (95:5, v/v). Effluents were evaporated to dryness, redissolved in 0.5 mL of methanol, and injected into the HPLC system.

RESULTS AND DISCUSSION

1-methyl-2-piperidinoethyl esters of phenylcarbamic acids are potential anaestethics.[18] Basic chromatographic parameters are described in Renčová's article.[20] The structure of studied compounds is shown in Figure 1.

Capacities of Polymers

The resultant values of polymer capacities are shown in Table 1.

The highest values of binding capacities were obtained using water for sample loading for all MIPs and also for NIPs. The whole amount of loaded analyte (100 μg) was sorbed onto the sorbents. In aqueous environments, hydrogen bonding and electrostatic interactions could be disrupted, and hydrophobic interaction, which are non-specific, could govern analyte retention.[21] This allows using water samples for extraction. Of course, after the sample loading, the cartridge should be dried

Table 1. Binding capacities of prepared polymers. RSD = 4.5–13.5%, $n = 3$

	Capacity (μg of analyte/100 mg of polymer)					
Solvent	MIP1	NIP1	SBC*	MIP2	NIP2	SBC?
Acetonitrile	2.5	0.4	2.1	20.4	10.7	9.7
Methanol	0.5	0.4	0.1	20.2	5.9	14.3
Water	>100	>100		> 100	> 100	

*The specific binding capacity was calculated by the deduction of the amount non-specifically adsorbed on NIP from the amount adsorbed on MIP.

and washed with selective organic solvent able to disrupt the non-specific interaction of analyte with polymer.

The capacity of MIP1 in methanol is low (prepared with acrylamid in acetonitrile) and the value of the capacity of MIP1 is comparable with the capacity value of NIP1. It means that there is no specific binding between imprinted polymer and template molecule. It can be caused by stronger interaction between methanol and analyte (4-DPCA) than between polymer and analyte. Methanol is a polar solvent, which can influence the hydrogen bonding between analyte and monomer. The capacity of MIP1 in acetonitrile is higher then that of NIP1. Acetonitrile was used as a porogene during the polymerization. Therefore, the best specific recognition was achieved in this solvent.

Unlike MIP1, the value of capacity of MIP2 (prepared with methacrylic acid in acetonitrile) in methanol is comparable to the value in acetonitrile. The interacions between functional groups of polymer and target molecule is strong enough in both solvents. Polarity and functional groups of monomer probably play important roles during polymerization.

Selectivity of MIPs

The selectivity of MIPs was tested towards two structurally related esters of alkolyphenylcarbamic acid with different length and position of the alkoxy-group. The influence of the length of alkoxy-chain on the polymer capacity was tested using the 1-methyl-2-piperidinoethyl ester of 4-methoxyphenylcarbamic acid (4-MPCA). The influence of the position of the alkoxy-chain was investigated by measuring of MIPs' capacity for 1-methyl-2-piperidinoethyl ester 2-decyloxyphenylcarbamic acid (2-DPCA). The polymer capacities of MIPs and relevant NIPs are shown in Table 2.

As is obvious from Table 2, the capacity of MIP1 is lower for an analyte with a shorter alkoxy-chain (4-MPCA) than for the template.

Table 2. Binding capacities of prepared polymers. RSD = 4.5–9.1%, $n = 3$

	Capacity (μg of analyte/100 mg of polymer)					
Solvent	MIP1	NIP1	SBC*	MIP2	NIP2	SBC*
4-DPCA (template)	2.5	0.4	2.1	20.2	5.9	14.3
4-MPCA	1.3	0.3	1.0	18.1	3.2	14.9
2-DPCA	1.9	0.4	1.5	10.2	6.2	4.0

*The specific binding capacity was calculated by the deduction of the amount non-specifically adsorbed on NIP from the amount adsorbed on MIP.

Therefore the length of alkoxy-chain impacts the capacity of sorbent for this analyte and MIP1 can recognize the template molecule from structurally related compounds with shorter alkoxy-chains. In the case of MIPs prepared from methacrylic acid (MIP2) the influence of alkoxy-chain length wasn't observed.

The influence of the alkoxy-chain position on the capacity of MIPs was also tested. In the case of MIP1, the difference between capacity values of the template (4-DPCA) and the analyte with a decyloxy-chain in orto-position (2-DPCA) is not very significant. But in the case of MIP2, the capacity of MIP is two times higher for the template than for the analyte with the methyloxy-chain in orto-position (4-MPCA), so MIP2 can recognize the template from structurally related compounds with alkoxy-chains in other positions.

Washing Step and Elution

The different washing and elution solvents were studied. Cartridges were conditioned with 5 mL of methanol and with 5 mL of solvent, which was used in the washing step. After the conditioning, the solution of 4-DPCA was added into a polymer catridge. Methanol, acetonitrile, water, mixture of methanol/acetic acid (99:1, v/v), mixture of methanol/acetic acid (95:5, v/v), and mobile phase were used as washing solvents. Each effluent was collected in 1 mL fractions. In the case of mixtures of methanol/acetic acid, all fractions were evaporated to dryness and redissolved in methanol. All fractions were analysed by HPLC. Results are shown in Figures 2–5.

According to the literature, the same solvent used for sample loading can be used in the washing step as well.[17] Acetonitrile, which was chosen for sample loading, was also tested as a washing solvent. Water was another solvent tested. It could be used for removing matrix components of hydrophilic properties from the cartridge. After the gradual application of water or acetonitrile into the MIP1, (Figure 2), no analyte

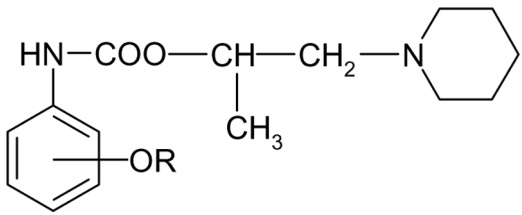

Figure 1. The structure of compounds used in the research. Template molecule: R = $-C_{10}H_{21}$ in 4-position (4-DPCA). Other analytes used in research: R = $-C_{10}H_{21}$ in 2-position (2-DPCA), R = $-CH_3$ in 4-position (4-MPCA).

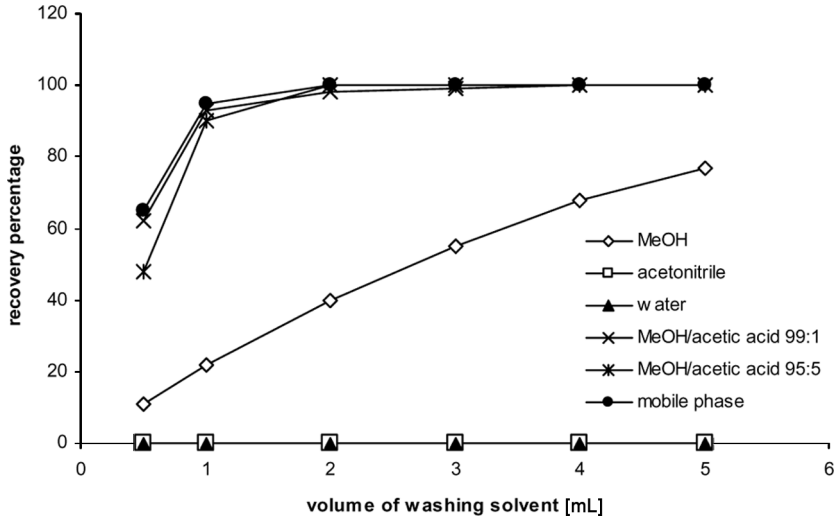

Figure 2. Release percentage of 4-DPCA after percolation of increasing volumes of different washing solvents through the MIP1.

was washed from the cartridge. This means that recovery is low for these solvents and they could be used as washing solvents. However, when acetonitrile was gradually added into NIP1, the whole amount of

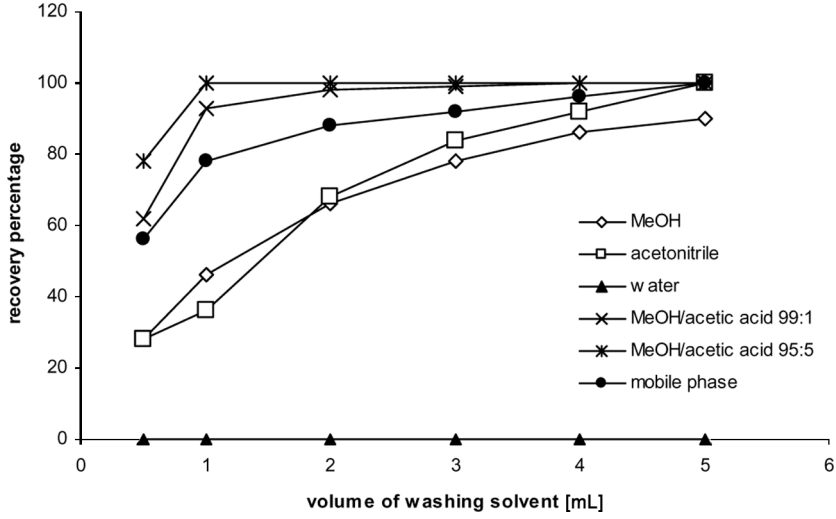

Figure 3. Release percentage of 4-DPCA after percolation of increasing volumes of different washing solvents through the NIP1.

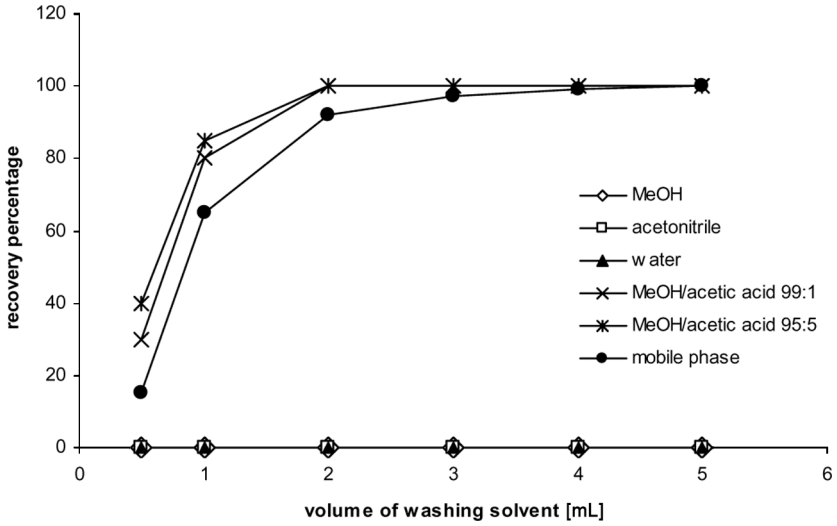

Figure 4. Release percentage of 4-DPCA after percolation of increasing volumes of different washing solvents through the MIP2.

4-DPCA was washed out from the cartridge with 5 mL of acetonitrile (Figure 3). It proves that molecules of the template were non-specifically adsorbed on the NIP1. After methanol application into MIP1, about 77%

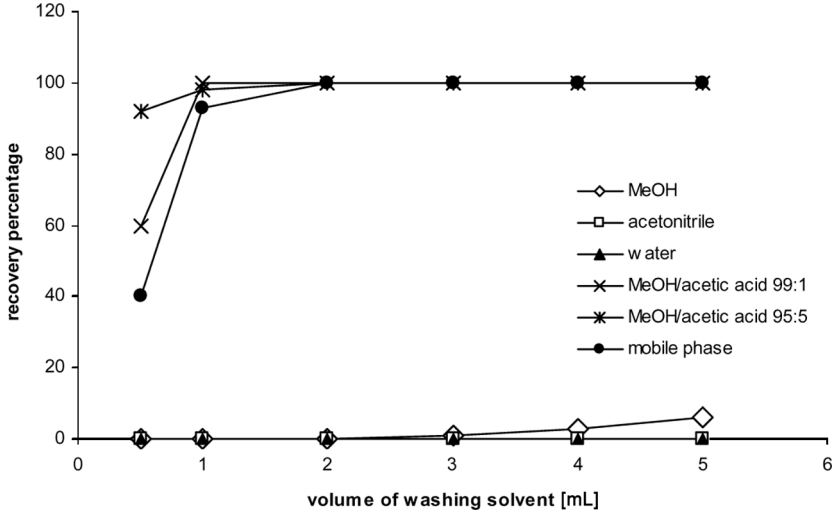

Figure 5. Release percentage of 4-DPCA after percolation of increasing volumes of different washing solvents through the NIP2.

of analyte was washed from the MIP1. Methanol probably formed stronger hydrogen-bonding with analyte than the polymer–analyte. When the mixtures of methanol and acetic acid (1% and 5% acetic acid in methanol) were used for the cartridge washing, the whole amount of loaded analyte was washed out from cartridge in small volumes (up to 2 mL).

After washing of MIP2 with water, methanol, and acetonitrile, respectively, no analyte was washed out from cartridge. Similar results were achieved for NIP2. This indicates that the interaction between the analyte and polymer are stronger than between analyte and these solvents. When the mixture of methanol and acetic acid was used the results were similar to MIP1.

All mentioned solvents were tested as the washing solvents also for the template's structurally related compounds (4-MPCA and 2-DPCA, respectively). The dependence of release percentage of studied esters of alkoxyphenylcarbamic acids on the volume of washing solvents are presented in Figures 6–9.

It can be seen from Figures [6–9] that there wasn't an observed loss of studied analytes (4-MPCA and 2-DPCA) from the cartridge during the washing of the cartridges by water and acetonitrile. Only a small amount of 2-DCPA was washed out by acetonitrile from MIP1. Methanol washed out 84% of 2-DPCA and 53% of 4-MPCA from MIP1. In the case of the mobile phase and the mixtures of methanol and acetic acid, the whole amount of both target analytes was washed out.

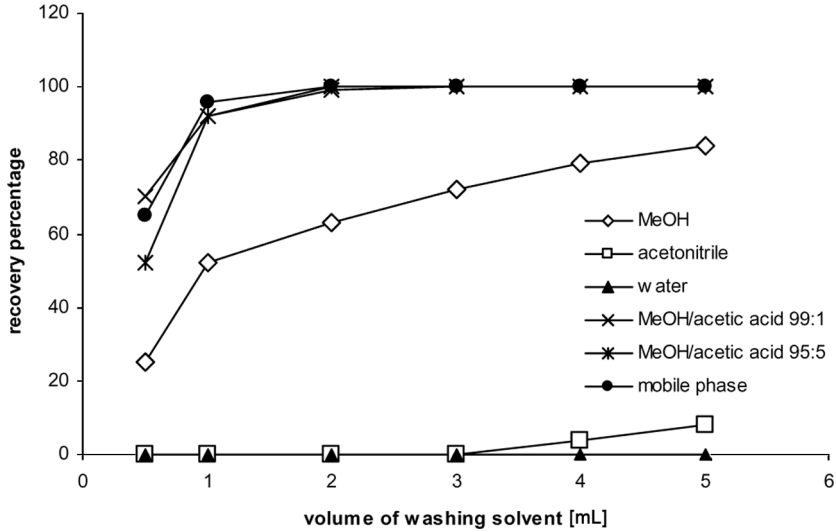

Figure 6. Recovery percentage of 2-DPCA after percolation of increasing volumes of different washing solvents through the MIP1.

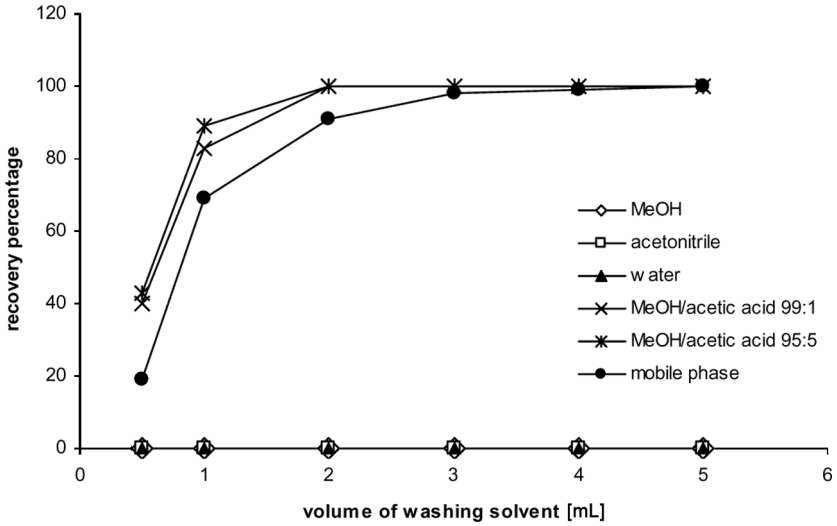

Figure 7. Recovery percentage of 2-DPCA after percolation of increasing volumes of different washing solvents through the MIP2.

Isolation of Analytes from Human Plasma

Matrix effects of spiked human plasma by using different sorbents (C_{18} and MIPs) for SPE were investigated. Percentage recoveries of studied

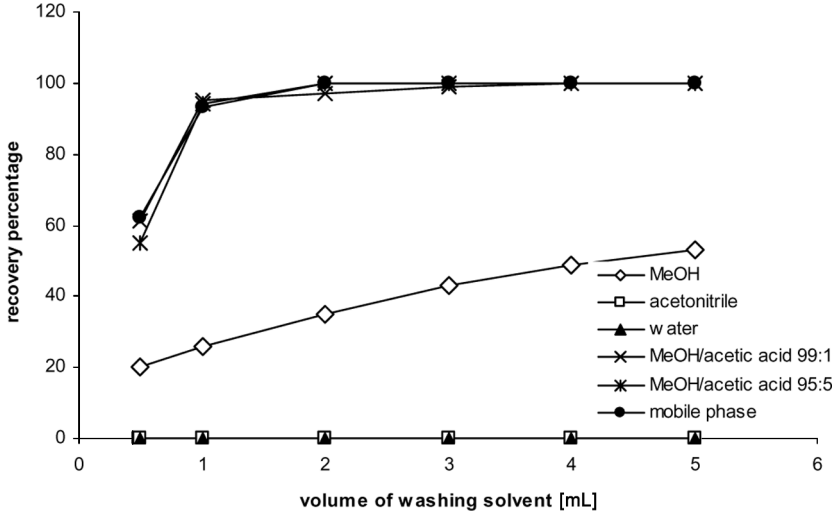

Figure 8. Recovery percentage of 4-MPCA after percolation of increasing volumes of different washing solvents through the MIP1.

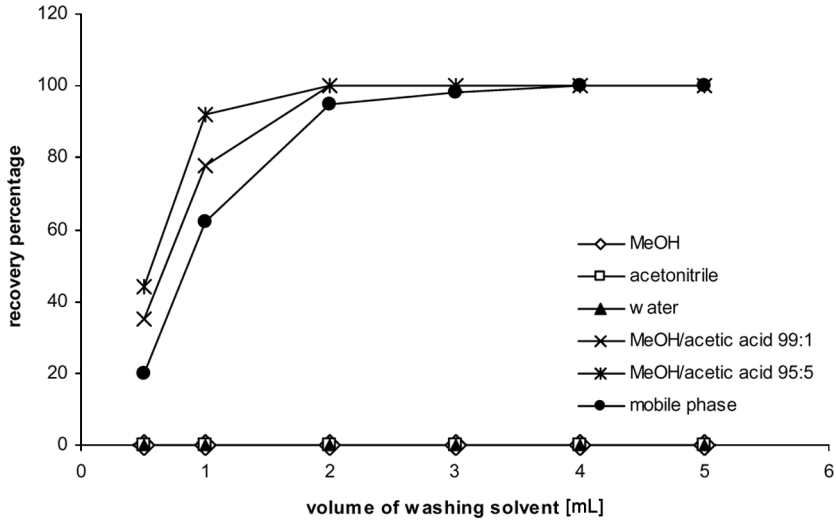

Figure 9. Recovery percentage of 4-MPCA after percolation of increasing volumes of different washing solvents through the MIP2.

analytes after SPE using the MIP1, MIP2, and C_{18} cartridge are shown in Table 3.

As is obvious from Figure 10 and Table 3, the recovery of 4-DPCA (template) is very good using MIP2 and C_{18}. The recovery of 2-DPCA and 4-MPCA is lower then for the template. Resultant recoveries of tested analytes are similar for both mentioned sorbents, the difference is in the presence of unknown compound interference from plasma eluted after 4-MPCA. The peak of this compound is higher in the chromatogram of extract after SPE on C_{18} than on MIP2. In this way the interferences can be minimized by using MIP2. In the case of MIP1, the recovery of each analyte is very low and it is not recommended for use in SPE.

Table 3. Recoveries of target analytes on investigated sorbents (%). RSD = 5–8%

	4-DPCA (template)	2-DPCA	4-MPCA
MIP1	12	3	9
MIP2	104	68	79
C18	102	67	80

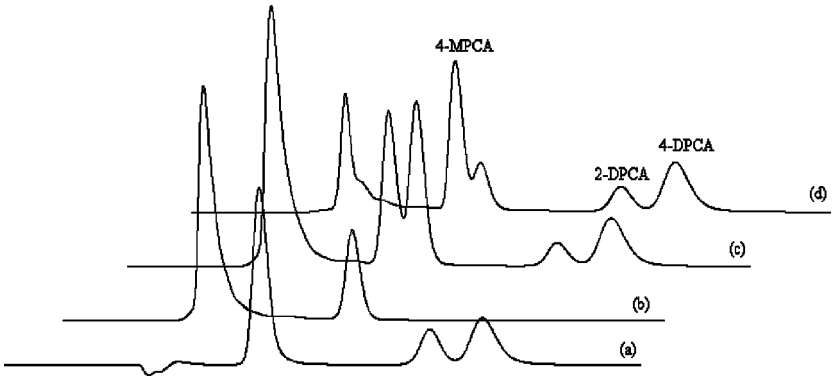

Figure 10. Chromatograms of mixture of standards (solution in methanol, c = 10 μg/ml) (a), blank human plasma after SPE on MIP2 (b) spiked human plasma after SPE on traditional C_{18} cartridge (c) and spiked human plasma after SPE on MIP2 (d). HPLC column: Separon SGX C18. Mobile phase: methanol/acetonitrile/acetic acid/diethylamine (80/20/0.1/0.1, v/v/v/v), izokratic elution. $F = 0.5$ mL/min. Detection: DAD, 240 nm. Injected volume: 50 μL.

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