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Determination of methotrexate in human serum by high-performance liquid chromatography combined with pseudo template molecularly imprinted polymer

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ABSTRACT

A pseudo template molecularly imprinted polymer (MIP) was prepared for methotrexate (MTX) and a RP-HPLC method combined with the MIP was developed for the determination of MTX in human serum. Because of the poor solubility of MTX in common MIP preparation solvents, trimethoprim (TMP), a molecule having the similar imprinting sites as MTX, is selected as the pseudo template. The MIP was prepared using methacrylic acid (MAA) and ethylene glycol dimethacrylate as functional monomer and cross-linker, respectively. ^{1}H NMR study showed highly strong interaction between TMP and MAA with hydrogen bonds. Chromatographic behaviors indicated that the TMP-MIP possessed excellent affinity and selectivity for MTX. And the imprinting factor for MTX was high up to 9.5 when 7:3 of acetonitrile:methanol (v/v) was used as mobile phase. Moreover, TMP-MIP was used as the solid-phase extraction (SPE) material to enrich the target compound MTX in human serum samples for HPLC analysis. The SPE process was carefully optimized and good recoveries of MTX were obtained as 81.6–86.2% with RSD of 0.22–1.84% when the spiked concentration of MTX was 2.0–10.0 μg mL^{-1} in human serum samples. The results indicated that the pseudo template MIP can be applied to preconcentration, purification and analysis of MTX in clinic samples.

1. Introduction

Methotrexate (MTX; 2,4-diamino-*N*10-methylpteroylglutamic acid) is a prototype folate antagonist cytotoxic drug and shows significant antitumor activity, which has been mainly used in the treatment of acute leukemia, bone cancer and other neoplastic diseases [1]. As a chemotherapeutic drug for tumors, MTX is usually used in high doses for acquiring a better treatment. However, the high-dose treatment may cause some side effects, such as vomiting, diarrhea, stomatitis, and myelosuppression, as well as hepatotoxicity later [2,3]. So the in vivo concentration of MTX of the patients must be carefully monitored. Some methods based on immunological techniques, such as fluorescence polarization immunoassay, radioimmunoassay, enzyme immunoassay, have been developed for the determination of MTX in plasma and serum [4–6]. However, endogenous factors, other disease states and haemolysis of samples interfered with the results. The HPLC methods [7–10] combined with a solid-phase extraction (SPE) process have also been developed for MTX determination. However, the commonly used SPE material is reversed-phase C_{18} packing, which lacks of selectivity to the target molecule. While MTX was enriched, the MTX metabolites and other interferers that existed in serum and plasma were also concentrated. In this case, a specific pretreatment material with better selectivity for target molecule is necessary.

Molecularly imprinted polymer (MIP) [11,12], an artificial recognition material with high affinity and selectivity to the template molecule, appears to be adequate to accomplish these demands. When MIP is used as SPE material, the target molecule can be enriched selectively from complex matrix. Since Sellergren [13] firstly utilized molecularly imprinted SPE (MISPE) method for enrichment and analysis of pentamidine from the urine sample, MISPE has been widely applied in the analysis of biological and pharmaceutical samples [13–15], as well as environmental samples [16–18].

Up to now, although many small molecules, such as drugs and amino acid derivatives, have been successfully imprinted, the MIP for other small molecules, such as toxic, expensive, unstable and poorly soluble targets, is still difficult to be achieved. Furthermore, when the MIP is used for trace analysis, template leakage will severely influence the accuracy of detection. Pseudo template, a compound which has the same or similar imprinting sites and spatial structure with the target molecule, is an effective substitute to solve these problems [19–22].

Due to the poor solubility of MTX in the common solvents of MIP preparation, the studies using several related compounds as pseudo templates for MTX recognition were performed [23–26]. However, the application of the MIP as SPE material combined with RP-HPLC for the MTX detection in human serum has not

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been reported. In this study, a MIP for MTX was prepared using trimethoprim (TMP) as the pseudo template and, a simple RP-HPLC method coupled with MIPSE process using TMP-MIP as SPE material was developed for the serum samples analysis. Firstly, TMP-MIP was polymerized using methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA) and chloroform as monomer, cross-linker and solvent, respectively. And then the chromatographic behaviors and selective recognition properties of MTX on the TMP-MIP column were evaluated. Furthermore, MTX in human serum sample was enriched selectively by the TMP-MIP and determined by RP-HPLC.

2. Experimental

2.1. Chemicals

Methotrexate (MTX), sulfamethoxazole (SMO), sulfamethazine (SMZ) and folic acid were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Trimethoprim (TMP) was a gift from Fukang Pharmaceutical Company (Shouguang, China). Metsulfuron-methyl (MSM) and bensulfuron-methyl (BSM) were gifts from Jiangsu Changlong Chemical Plant (Changzhou, China). Methacrylic acid (MAA) and 2,2-azobis(2-isobutyronitrile) (AIBN) were purchased from Beijing Chemical Plant (Beijing, China). Ethylene glycol dimethacrylate (EDMA) was purchased from Sigma–Aldrich. MAA was distilled under vacuum to remove stabilizers prior to use. AIBN was recrystallized from ethanol before use. All other chemicals were of the analytical or the HPLC grade and used without further treatment.

2.2. Preparation of TMP imprinted and non-imprinted polymer

The pseudo template TMP (0.8 mmol), functional monomer MAA (4 mmol) and cross-linker EDMA (24 mmol) were dissolved in 15 mL of chloroform in a conical flask. After sonication for about 5 min, 50 mg AIBN was added as an initiator of the radical polymerization reaction. The mixture was purged with nitrogen for 5 min, followed by degasification under vacuum for 5 min and then sealed. The polymerization was carried out in a water bath maintained at 55 °C for 24 h. The obtained bulk polymer was ground to fine powders and subsequently sieved to obtain 25–40 μm particles. The TMP-imprinted particles were packed into a 100 mm × 4.6 mm i.d. stainless steel column, washed on-line with methanol:acetic acid (4:1, v/v) and then with methanol until no residue of TMP was found in the rinses. The non-imprinted polymer (NIP) for control experiments was prepared identically according to the process described above, except for the addition of TMP.

2.3. Chromatographic evaluation of MTX on the TMP-MIP column

Chromatographic investigations were performed on a Hitachi D-7000 HPLC system, which consists of a D-7000 interface, a L-7100 pump, a L-7420 UV–Vis detector, a L-7610 degasser, a L-7300 column oven and a Rheodyne 7725i sample injector. The data were acquired and processed with a D-7000 chromatographic workstation (Hitachi, Japan). Detection was performed at 306 nm. The retention factor (k) was calculated from the equation $k = (t_R - t_0)/t_0$, where t_R and t_0 are the retention times of retained solutes and void marker, respectively. The retention time of the void marker, t_0, was measured by injecting acetone. The imprinting factor (IF) was calculated from the equation $IF = k_{imprinted}/k_{non\text{-}imprinted}$.

2.4. MISPE process

The MISPE cartridges were prepared by packing 200 mg of the dry TMP-MIP and NIP into empty SPE-cartridges, respectively. Prior to use, the MIP and the NIP SPE cartridges were conditioned using 5 mL methanol, 3 mL acetonitrile and 5 mL water, respectively. MTX standard solutions or MTX spiked and pre-treated real serum samples were loaded onto the MISPE cartridges with a flow rate of 0.3 mL min^{-1}. After washing with 5 mL water, the cartridges were washed using different organic solvents. Finally, the cartridges were eluted with 5 mL of acetonitrile:methanol (3:7, v/v) at a flow rate of 0.4 mL min^{-1}. The eluted fraction was collected and immediately dried using a N_2 stream at 30 °C. The residues were dissolved in 200 μL of mobile phase and 20 μL of them were analyzed by RP-HPLC.

2.5. RP-HPLC analysis

The quantitative analysis of MTX was performed by RP-HPLC on the Hitachi HPLC system. An Ultimate C_{18} column (250 mm × 4.6 mm i.d., 5 μm, Shanghai, China) was used for the chromatographic separation. The detection wavelength was set at 306 nm. The mobile phase was methanol:0.05% H_3PO_4 aqueous solution (23:77, v/v) with a flow rate of 1.0 mL min^{-1}. Peak areas were used for quantification. The different concentrations of pure MTX solutions were prepared as 0.5, 1.0, 2.0, 5.0 and 10.0 μg mL^{-1}. The calibration curve (amount of MTX versus peak area) was prepared using pure MTX as the standard. The correlation coefficient of the standard graph was equal to 0.9998 (n = 4). According to the formula: recovery = (found/spiked) × 100%, the recovery of MTX from MISPE could be determined.

2.6. Serum samples preparation

0.5 mL of human serum spiked with MTX at different concentrations was firstly adjusted to pH value of 5.0. In order to avoid a great dilution of serum samples, 1.0 mol L^{-1} HCl solution instead of buffer solution was selected to adjust the pH value of serum samples. Very small amount of HCl solution was used and the change of serum volume could be neglected. After pH adjusting, the MTX serum samples were treated with 1.0 mL of methanol. After centrifugation, the supernatant was dried using a N_2 stream to eliminate the methanol. The residue was redissolved in water and loaded onto the MISPE and NISPE cartridges according to the process described in Section 2.4.

3. Results and discussion

3.1. MIP preparation and its molecular recognition properties

MTX is very difficult to imprint because of its poor solubility in the common MIP preparation solvents, such as acetonitrile, chloroform and toluene. So, a pseudo template method was tried to overcome this problem in this study. Fig. 1 shows the structures of the target molecule MTX and its pseudo template TMP. Clearly, the TMP molecule has the same group, 2,4-diamino pyrimidinyl, with the MTX, which is the most important group during the imprinting and recognition processes. And also the spatial structure near the imprint sites of TMP is similar with MTX. Furthermore, MTX and TMP can be easily separated by C_{18} column (data not shown) and will not interfere with the accuracy determination of MTX even if there is small amount of TMP leakage. The schematic of imprinting and recognition process is shown in Fig. 2. TMP and MAA form a complex by self-assembling firstly, and then polymerize with EDMA by a bulk polymerization method to produce the TMP-MIP. Because of the similar imprinting and recognition properties, the obtained TMP-MIP may have excellent molecular recognition ability for MTX.

^{1}H NMR spectra analysis, which was performed to study the intermolecular interaction, was used to investigate the imprinting mechanism [27]. ^{1}H NMR spectra of TMP was shown in Fig. 3A

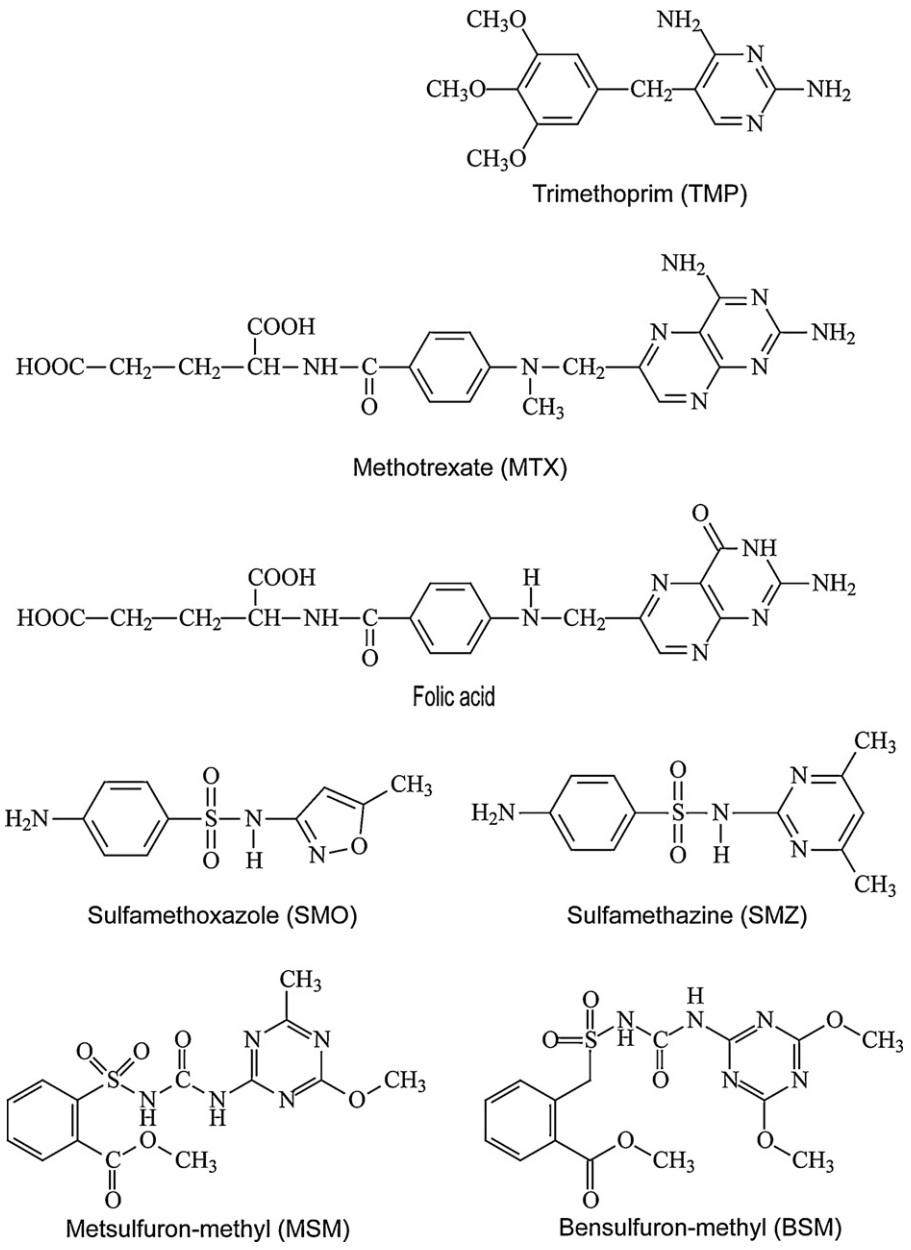

Fig. 1. Molecular structures of TMP, MTX and other compounds used in this study.

(400 MHz, $^{\delta-}$CDCl$_3$, δ 3.65 (–CH$_2$–), δ 3.81 (–OCH$_3$), δ 4.59 (–NH$_2$), δ 4.78 (–NH$_2$), δ 6.38 (benzene, H-2, 6), δ 7.78 (pyrimidine, H-6)), the chemical shift of the two amino groups of TMP was 4.59 and 4.78, respectively. When functional monomer MAA (400 MHz, $^{\delta-}$CDCl$_3$, δ 1.94 (–CH$_3$), δ 5.63, 6.20 (=CH$_2$), δ 12.84 (–COOH)) was added, the two peaks of amino groups of TMP disappeared (Fig. 3B), which indicated that strong interaction of hydrogen bonds between the amino group of TMP and the carboxyl group of MAA was formed. When the target molecule MTX is treated with pseudo template MIP materials, the hydrogen bond between MTX and MAA will also emerge due to the similar binding site and spatial structure of MTX and TMP.

In order to confirm the molecular recognition ability of the TMP-MIP material for MTX, the retention behaviors of MTX on TMP-MIP and NIP columns were studied. From Fig. 4, it can be clearly seen that the retention of MTX on the TMP-MIP column were much stronger than that on the NIP column when a different ratio of acetonitrile to methanol was used as mobile phase. Especially when the ratio of acetonitrile to methanol was 9:1 (v/v), a very long retention of MTX on the TMP-MIP was obtained, whereas the corresponding retention factor of MTX on the NIP was only 3.78. And the imprinting factor (IF) values were 9.5, 4.4, 3.9 and 3.7 when the ratio of acetonitrile:methanol was 7:3, 5:5, 3:7 and 0:10, respectively. The results indicated that the TMP-MIP possesses excellent molecular recognition ability for MTX. Additionally, very strong retention of MTX occurred on both TMP-MIP and NIP columns when water was used as the mobile phase (data not shown), which indicated that the real aqueous samples containing MTX can be loaded onto the MISPE column directly for MTX selective adsorption.

3.2. Binding specificity of TMP-MIP for MTX and its analogs

A proper pseudo template of target compound is very important for imprinting. In order to study the binding specificity of TMP-MIP, some related compounds of SMO, SMZ, MSM, BSM and folic acid were selected and their retention behaviors on the TMP-MIP were also investigated. The chromatogram was shown in Fig. 5. All the selected compounds were not retained on the TMP-MIP col-

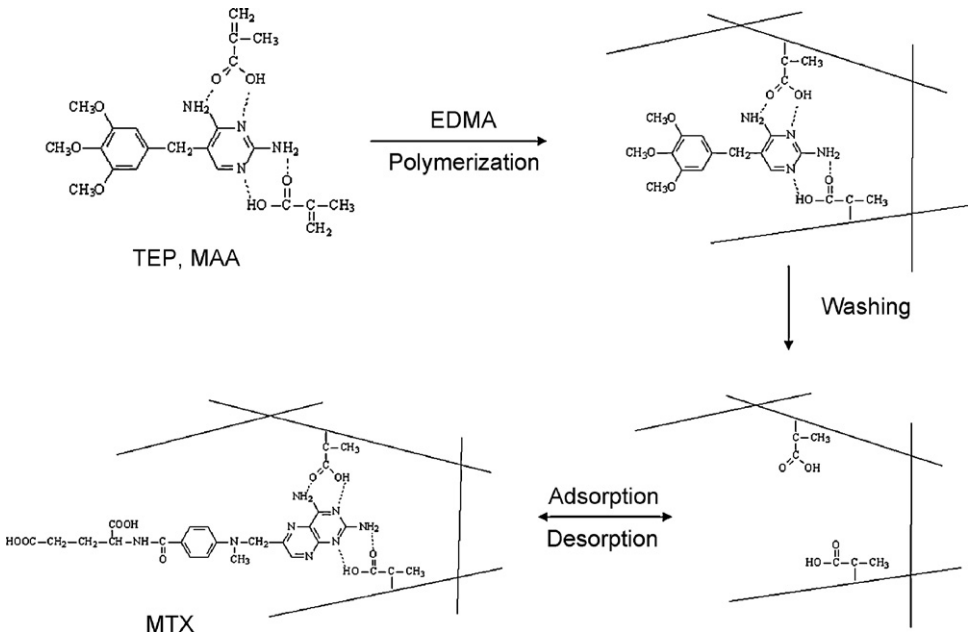

Fig. 2. Simplified schematics of imprinting procedures.

umn. From Fig. 1, it can be found that all these compounds have nitrogen heterocycles. However, the substitutional groups of the nitrogen heterocycles are methyl and methoxyl groups on SMO, SMZ, MSM and BSM whereas amino groups on TMP and MTX. This led to the recognition sites of TMP-MIP are not compatible with these four molecules and also hydrogen bonds, the main molecular recognition force, cannot be formed. The results indicated that the amino group on the nitrogen heterocycle of TMP and MTX played an important role in imprinting and recognition process. Furthermore, folic acid, a very similar molecule with MTX, was also not retained on the TMP-MIP. The reason may be that an amino group of nitrogen heterocycle on MTX was changed to a carbonyl group on folic acid, which causes the major changes of polarity and acid–base character of these two molecules, resulting in the hydrogen bonds cannot be formed and the folic acid cannot be recognized on the TMP-MIP.

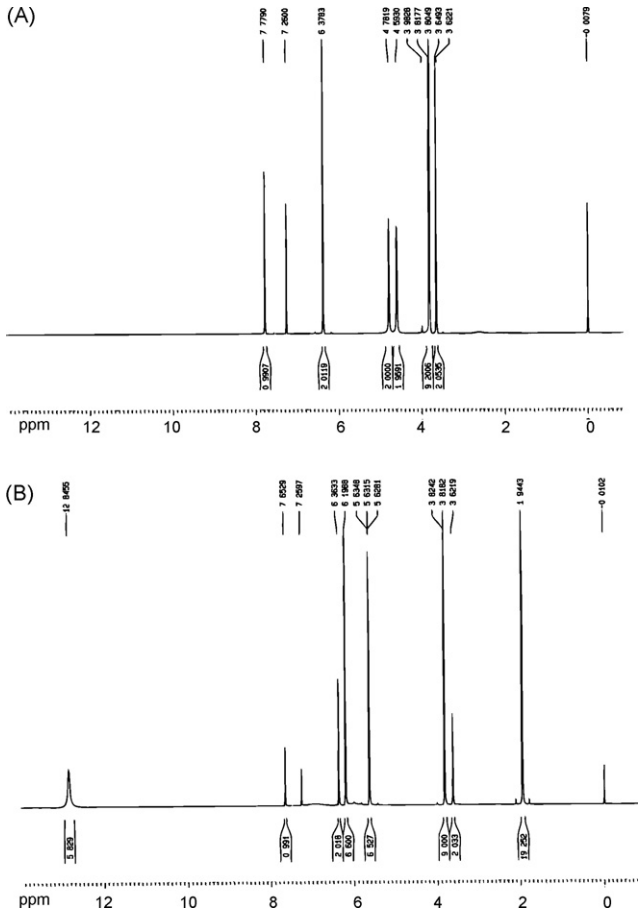

Fig. 3. ^{1}H NMR spectra of TMP (A) and TMP-MAA (B).

3.3. Optimization of MISPE conditions for MTX enrichment

MIP based SPE can selectively enrich the object which was essential for analyzing the complex samples, such as serum, plasma or

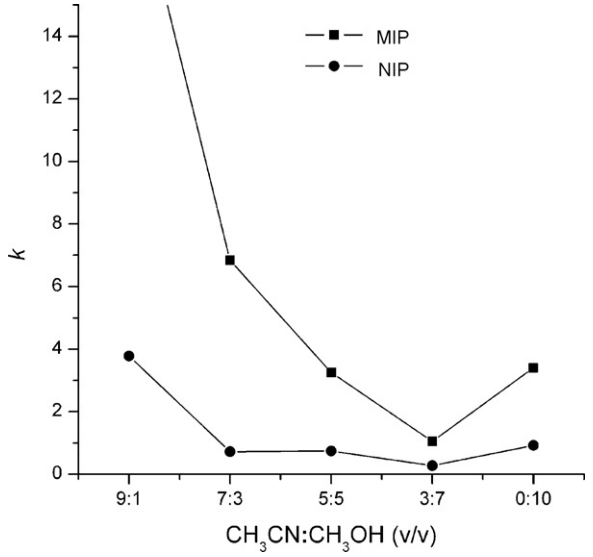

Fig. 4. Effect of different mobile phase on the retention factor k of MTX on TMP-MIP and NIP columns. HPLC conditions: column size, 100 mm × 4.6 mm i.d.; mobile phase, acetonitrile:methanol; flow rate, 1.0 mL min^{-1}; detection, 306 nm.

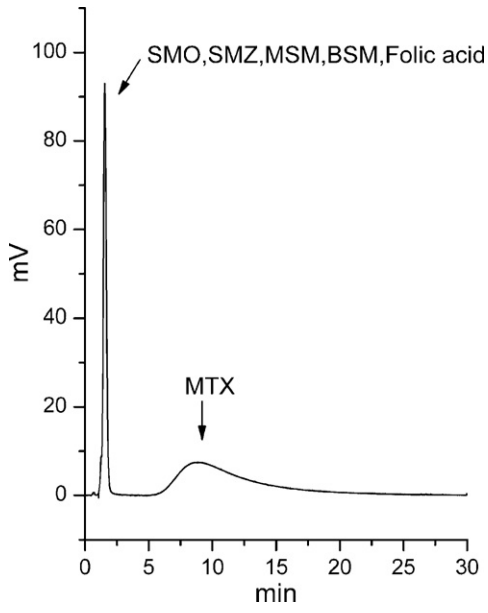

Fig. 5. Separation of MTX and SMO, SMZ, MSM, BSM and folic acid on the TMP-MIP column. HPLC conditions: column size, 100 mm × 4.6 mm i.d.; mobile phase, acetonitrile:methanol (7:3, v/v); flow rate, 1.0 mL min^{-1}; detection, 306 nm.

environmental samples. Washing and elution solvents were important elements for establishing an optimum MISPE protocol. Solvents should be low-cost, low-toxicity and should make the MISPE with high selectivity, high recovery. According to Fig. 4, when acetonitrile:methanol (3:7, v/v) was used as the mobile phase, a very short retention on the MIP was obtained, and then it was selected as the elution solvent in the MISPE process.

Washing solvent serves an important role to remove the contaminants and retain the analytes on the column during the SPE process. In this study, acetonitrile, chloroform:acetonitrile (5:95, v/v), chloroform:acetonitrile (10:90, v/v) and chloroform were used as the candidates for washing solvent. Firstly, 1 mL of 200 ng mL^{-1} of MTX aqueous solution samples was loaded on the SPE cartridge, and then 4 mL of washing solvent flowed through the cartridge to remove the impurities. Next, the enriched MTX on the SPE cartridge was eluted and dried using a N_2 stream. The residues were redissolved and analyzed by RP-HPLC. As known, water and chloroform are not mutually mixable. In order to avoid the influence of unmixing between chloroform and water on MTX adsorption and elution, the SPE cartridge was vacuumed to remove water after the serum sample was loaded. And then chloroform or other solvents were added to wash the cartridge. In this case, the unmixing of water and chloroform cannot disturb the washing step and further detection. The experimental results were shown in Fig. 6. Clearly, the recovery of MTX on the TMP-MISPE was high from 79% to 95% in four kinds of washing solvents, which showed the excellent affinity and recognition ability of TMP-MIP for MTX. However, the recovery of MTX on the NISPE changed greatly from 26% to 82% along with the increase of chloroform content. When 100% chloroform was used as washing solvent, a high recovery was obtained on the NIP, revealing the non-specific adsorption for MTX. Acetonitrile can successfully destroy the non-specific adsorption, and a very low recovery of 26% for TMX was obtained on NISPE when 100% acetonitrile was used as washing solvent. When the content of chloroform was 5%, the recoveries of MTX was low (36%) on the NISPE and high (90%) on the MISPE. So, 5% chloroform in acetonitrile was selected as washing solvent in the further study.

3.4. Human serum analysis

Human serum samples spiked with different concentration of MTX were analyzed by the developed MISPE extraction and RP-HPLC analysis. As control experiments, NISPE for MTX spiked serum sample and MISPE for non-MTX spiked serum sample were also investigated in the same conditions. The extracted fractions were analyzed by RP-HPLC and the chromatogram was shown in Fig. 7. The simple chromatograms illustrated that almost all the interferences existed in human serum were removed after being treated with SPE. Furthermore, when no MTX was spiked to the serum sample, no peak occurred on the chromatogram (Fig. 7A). If the MTX spiked serum sample was treated with NISPE, only 16.3% of MTX was recovered from the NISPE column and a small peak was seen in Fig. 7B. However, when the same spiked serum sample was extracted on the TMP-MIP column, more than 80% of MTX was recovered from the column and a big peak was shown in Fig. 7C. These phenomena further confirmed the specific recognition of TMP-MIP to MTX. The serum samples spiked with different concentration of MTX were measured and the results were shown in Table 1. It can be inferred that when the concentration of MTX was increased from 2.0 to 10.0 μg mL^{-1}, the recoveries were 81.6–86.2%

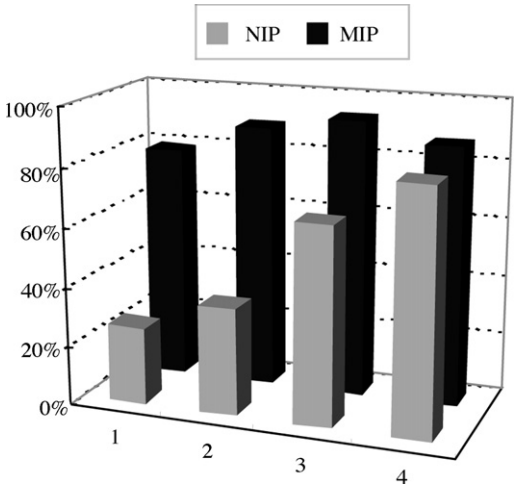

Fig. 6. The recovery of MTX on the TMP-MISPE and NISPE after washing with 4 mL of different solvents: (1) acetonitrile, (2) chloroform:acetonitrile (5:95, v/v), (3) chloroform:acetonitrile (10:90, v/v), and (4) chloroform.

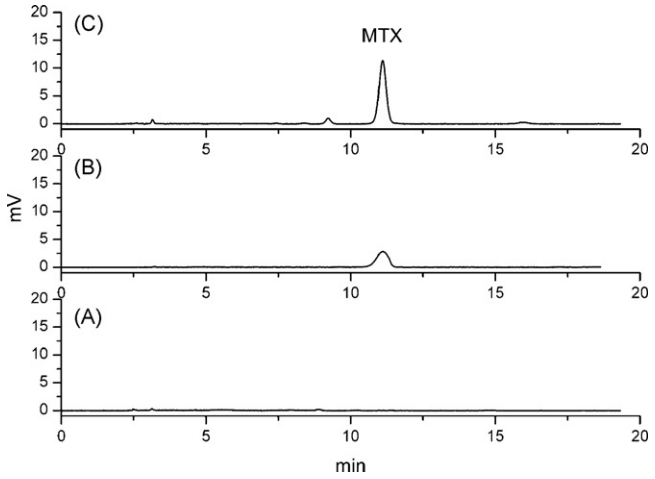

Fig. 7. Chromatograms (RP-HPLC) obtained by (A) MISPE, no MTX spiked, (B) NISPE, 2.0 μg mL^{-1} of MTX spiked, and (C) MISPE, 2.0 μg mL^{-1} of MTX spiked. HPLC conditions are shown in Section 2.5.

Table 1
Recoveries of MTX obtained from human serum samples.

Concentration ($\mu g\,mL^{-1}$)	Spiked MTX (μg)	Determined MTX (μg)	Recovery (%)	RSD (%, $n = 3$)
1.00	0.50	0.33	66.2	0.31
2.00	1.00	0.82	81.6	0.89
5.00	2.50	2.16	86.2	0.22
10.0	5.00	4.13	82.5	1.84

with RSD of 0.22–1.84% ($n = 3$). These results demonstrated that the RP-HPLC analysis combined with MISPE extraction can be used for the analysis of MTX in the serum samples with good accuracy and precision.

4. Conclusions

A pseudo template method for MIP preparation was reported and a MIP of MTX was prepared using TMP as the pseudo template. The TMP-MIP shows good molecular recognition ability and selectivity for MTX, whereas some analogs, such as folic acid, SMO, SMZ, MSM, BSM, were not retained on the TMP-MIP, indicating that the same or homologous imprinting sites and spatial structure were very important for imprinting and recognition. Moreover, the RP-HPLC analysis combined with MISPE extraction was successfully applied to determine the MTX in human serum samples with good recovery and reproducibility.

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