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A novel restricted access material combined to molecularly imprinted polymers for selective solid-phase extraction and high performance liquid chromatography determination of 2-methoxyestradiol in plasma samples

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ABSTRACT

A feasibility study was performed in order to ensure the possibilities in using a restricted access material combined to molecularly imprinted polymers (RAM-MIP) as sorbent material in solid phase extraction (SPE) for clean-up of 2-methoxyestradiol (2-ME) from plasma samples. The MIP with hydrophilic external layer was designed by precipitation polymerization. The polymer was characterized by thermogravimetric analysis (TGA) and scanning electron microscope (SEM). The use of analogs of 2-ME as templates, in combination with a chromatographic separation of the analytes in the sample, overcame the problem of the template bleeding. To demonstrate the property of the RAM-MIP obtained, a comparison of commercially available C_{18} SPE was performed. The results showed that the RAM-MISPE recoveries were significantly higher than that of C_{18} SPE for 2-ME in trace concentration. During the extraction process, 2-ME was sufficiently cleaned for further chromatographic analysis with no interferences from template leakage and matrix. Good linearity was obtained from 0.06 to 20 μg mL^{-1} with the correlation coefficient $r > 0.9991$. The coefficient of variation of the inter-assay precision was less than 11.9%. The recoveries of 2-ME in rat plasma at three spiked levels were in the range of 99.10–101.00%. Based on the analytical validation results, the proposed method (RAM-MIP off-line SPE/HPLC) can be a useful tool to determine 2-ME in rat plasma samples.

1. Introduction

For the liquid chromatographic determination of drugs and their metabolites in plasma, tedious and time-consuming pre-treatment procedures such as the removal of proteins by precipitation, liquid–liquid extraction or solid phase extraction (SPE) have been required in the past. Recently, restricted access matrix-solid phase extraction (RAM-SPE) has attracted increasing attention for the direct extraction of analytes from biological fluid [1,2]. With RAM large molecules such as proteins are eluted in the void volume without destructive accumulation because of restricted access to some surfaces, while allowing small molecules such as drugs to reach the hydrophobic, ion-exchange or affinity sites and to be separated [3]. However, the RAM developed could not be used for selective enrichment of an analyte, because of its separation mechanism. On the other hand, the molecularly imprinted polymers (MIPs), which can afford specific recognition against an imprint molecule and moderate recognition against the structurally related compounds, are used for SPE. Since Sellergren1 [4] firstly reported the use of MIP as SPE sorbents, a wide range of analytes of biological, pharmaceutical, food and environmental samples have been involved [5–7]. However, protein adsorption negatively interfered with their recognition properties [8]. To overcome this difficulty, numerous techniques have been used to deproteinize biological fluids before analysis, which is more time-consuming and can add sample artifacts [9,10]. The development of a special and selective extraction support, allowing the direct cleanup of biological samples, is required.

Recently, the RAM combined to molecularly imprinted polymers (RAM-MIP) was developed. Owing to the hydrophilic modification of MIP surface, the materials avoid the destructive deposition of biomacromolecules on the polymeric surface [11,12]. Puoci [13] has obtained RAM-MIP for selective recognition of *p*-acetaminophenol in gastrointestinal simulating fluids. But whether the RAM-MIP can be directly applied to pretreatment of the drug from plasma is not

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clear. The aim of our study was to prepare a RAM-MIP with hydrophobic inner surface and hydrophilic outer surface, which was a hydrophilic modification of MIP surface. The obtained RAM-MIP was used as an adsorbent for solid-phase extraction of 2-ME from the plasma.

2-Methoxyestradiol (2-ME), an endogenous metabolite of 17β-estradiol (Fig. 1), prevents the formation of new blood vessels, acts as a vasodilator and induces apoptosis in some cancer cell lines. Several analytical methods have been reported for the determination of 2-ME, including LC-UV linked solid-phase extraction [14], GC–MS [15], UPLC/QTOF-MS [16] and radioimmunoassay using a 125I-labeled ligand [17]. Although most of these methods are specific, their applications have been limited due to high cost, insufficient selectivity and time-consuming sample preparation. For its potential pharmaceutical applications and low blood drug concentration, the development of a simple, rapid, sensitive and selective method for the determination of 2-ME is therefore highly desirable.

In this study, the polymer was prepared by the precipitation polymerization and chemical modification [18]. We briefly described the synthetic routes and methods available to evaluate the polymers. RAM-MISPE protocol was optimized and applied for cleanup and enrichment of 2-ME from rat plasma samples. To date, the most successful way to avoid any unwanted leaching during pre-concentration resulting in clean and selective extraction of the analyte is the use of an analog of the target molecule during MIP design and production, known as "dummy molecularly imprinted polymer" (DMIP) [19]. The influence of template bleeding in trace analysis is avoided, since template used in the proposed non-covalent approach is only a close analog of analyte. The proposed method is a basis of online SPE (RAM-MIP as sorbents) in combination with HPLC.

2. Experimental

2.1. Chemicals and animals

Methyl methacrylate (MMA), ethylene glycol dimethacrylate (EGDMA), 2,2-azobisisobutyronitrile (AIBN) and glycidyl methacrylate (GMA) of reagent grade were purchased from Aldrich (Steinheim, Germany). 2-ME, estradiol, hydrocortisone acetate and glibenclamide were from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Their chemical structures are shown in Fig. 1. All the other reagents used in the experiment were obtained from Tianjin Shield Specialty Chemical Co. Ltd. (Tianjin, China). Age-matched Sprague-Dawley (SD) rats (250 ± 10 g) were obtained from the Zhengzhou University Medical Laboratory Animal Center (Zhengzhou, China).

2.2. Instrumentation and analytical conditions

HPLC analysis was performed on Agilent 1100 HPLC (Agilent, Palo Alto, USA) equipped with autosampler, thermostated-column device and a fluorescence detector. The chromatographic separations were carried out on a Dikma C_{18} column (200 mm × 4.6 mm, 5 μm), with a mobile phase consisting of acetonitrile–water–methanol (50:40:10, v/v/v), at a flow rate of 1.0 mL min^{-1}. Column temperature was maintained at 30 ± 1 °C. Aliquots of 20 μL were injected into the column and the chromatograms were recorded at excitation wavelength 285 nm and emission wavelength 325 nm. Agilent ChemStation® software was used for data acquisition and integration.

The approximate size distributions were determined by Nano-ZS90 laser nanometer particle sizer (Malvern, England). JSM-7500F scanning electron microscope (SEM) (Tokyo, Japan) and the thermogravimetric analysis (TGA) (Selb, Bavaria, Germany) were used for characterization of polymers. Solid phase extraction was performed using a Supelco 12-position SPE manifold and Supelclean LC-18 columns (Pennsylvania, Bellefonte, USA).

2.3. Preparation of the imprinted polymers

Estradiol (0.2 mmol), the analog of 2-ME, as the template molecule, was dissolved with 25 mL acetonitrile in a 50 mL glass tube fitted with a screw cap [20–23]. The functional monomer (MMA, 1.2 mmol) was then added. The tube was sonicated for 5 min and pre-polymerized at room temperature for 4 h to facilitate template–monomer complex formation. Thereafter, the co-monomer (GMA, 1.2 mmol), the cross-linking monomer (EGDMA, 6.0 mmol) and the

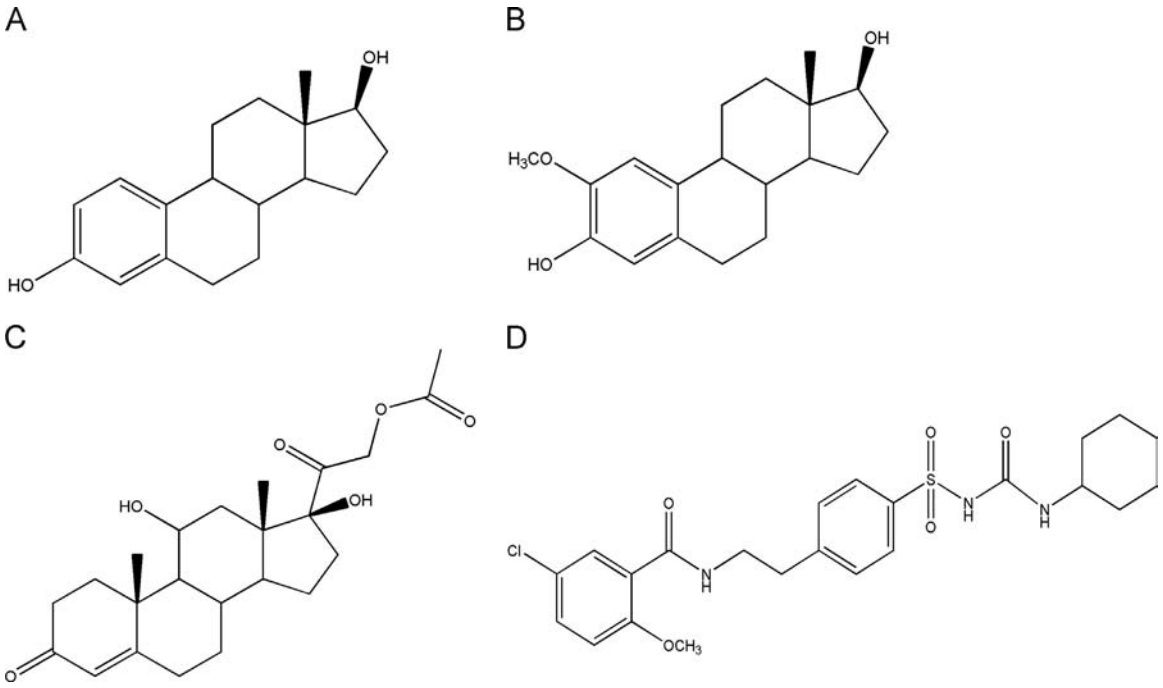

Fig. 1. The chemical structures of (A) estradiol, (B) 2-ME, (C) hydrocortisone acetate and (D) glibenclamide.

initiator (AIBN, 0.15 mmol) were added, and the resultant solution was cooled on an ice bath and degassed with nitrogen stream for 15 min before being sealed. The polymerization was carried out at 60 °C in a thermostatic water bath for 24 h. After reaction, the polymers were dried overnight in a vacuum oven at 40 °C.

The epoxy groups on the surface of polymers were targeted for chemical modification. The powder 400 mg was added to 25 mL of 10% perchloric acid solution in order to open epoxide ring, then agitated for 24 h at room temperature. At the end of the reaction, the particles were filtered, washed with 100 mL of ethanol, acetone, and diethyl ether. Finally, the template was removed by ultrasonic extraction, the RAM-MIP particles were obtained. The RAM non-imprinted polymers (RAM-NIP) were prepared as the same procedure, including washing, but with the omission of the template molecule. The MIP synthesized without GMA insertion was almost same as that of the RAM-MIP [24].

2.4. Binding experiments

Batch adsorption experiments were used to evaluate the binding affinity of the imprinted polymer as reported before [25,26], the adsorption ability of RAM-MIP and RAM-NIP were evaluated. 20 mg of RAM-MIP and RAM-NIP particles were added to a conical flask containing 10 mL of 2-ME water/methanol solution (95/5, v/v, 30 μg mL^{-1}), respectively. Samples were shaken at room temperature for 12 h. After that, aliquots of the supernatant were collected after centrifugation (10,000*g* for 10 min), and 2-ME was measured by HPLC. Experiments were repeated five times. The adsorption quantity (Q, μg mg^{-1}) of 2-ME was determined by following equation:

$$Q = (C_0 - C)V_0/m \quad (1)$$

where V_0 is the volume of solution (mL), m is the mass of RAM-MIP materials, C_0 and C are the initial and equilibrium concentration of 2-ME after the adsorption, respectively.

2.5. Protein binding

30 mg of dry particles of polymers were packed into a 1.0 mL polypropylene SPE column. Before use, the columns were preconditioned by successive washings with water, HCl (0.07 mol L^{-1}), water, methanol/water (50/50, v/v), water, and finally phosphate buffer (25 mmol L^{-1} pH 7.4). The adsorption test was performed by loading the cartridge with bovine serum albumin (BSA) standard solution in phosphate buffer. BSA was loaded (0.5 mL) four times consecutively. The amount of adsorbed protein after each loading was calculated at 290 nm of UV–vis spectrophotometer by coomassie brilliant blue staining (CBB) which is the basis of a popular method of protein assay. Experiments were repeated five times.

2.6. RAM-MISPE protocol for plasma samples

Extraction was performed by a 12-port vacuum extraction manifold with a water aspirator to produce vacuum. Empty SPE cartridges (1 mL) were packed with 30 mg corresponding RAM-MIP or RAM-NIP. Before each use, cartridges were pre-conditioned with 1 mL of acetic acid/methanol (5:95, v/v) and 2 mL methanol, followed by 2 mL water in order to remove potential contaminants, including any template still present in the imprinted material. The SPE extraction involved: loading with 500 μL plasma sample spiked with 2-ME, washing with 8 mL of methanol/water (5/95, v/v), followed by 3 mL of methanol/water (30/70, v/v) and eluting with 3 mL of acetic acid/methanol (1/9, v/v). After extraction, the extracts were evaporated to dryness at 40 °C under a stream of nitrogen and the residue was redissolved in the mobile phase and analyzed by HPLC. The RAM-MISPE variables such as the washing solution, eluting solution, and the speed of extraction were optimized, to improve the analytical sensitivity of the proposed method.

As a comparison experiment, the commercial C_{18} SPE cartridges were also performed for pretreating 2-ME-containing samples as reported [27]. The cartridges were conditioned with 2 mL of methanol and then equilibrated using 2 mL of water. 500 μL of plasma were applied to the column, and then rinsed with 2 mL of methanol/water (5/95, v/v). Elution was performed with 2 mL of methanol, which was evaporated to dryness under a continuous stream of nitrogen at 40 °C. The extracts were reconstituted in 100 μL of acetonitrile using vortex-mixing and 20 μL was injected into the HPLC.

2.7. Analytical validation

To optimize and validate the developed RAM-MIP method, drug-free plasma samples obtained from the orbital vein of the rats were used. The stock standard plasma sample was prepared by using blank plasma spiked with 2-ME at a concentration of 1 mg mL^{-1}. The working standard plasma samples were obtained by diluting the stock sample (1 mg mL^{-1}) in blank plasma, which resulted in 2-ME concentrations of 0.06, 0.1, 0.2, 1, 6, 16 and 20 μg mL^{-1}. These solutions were stable for five days, at 4 °C. Aliquots of 500 μL of the calibration standards and plasma samples were loaded onto the RAM-MIP SPE cartridges. The selectivity of the method was investigated by comparing the retention properties of 2-ME with those of endogenous compounds. The calibration curve was constructed by plotting the peak areas of the analytes versus their concentrations. The intra- and inter-day precision data were obtained with the assay of spiked samples. Accuracy values were calculated by comparing the concentrations of 2-ME added to the plasma samples. The reusability and robustness were also evaluated.

3. Results and discussion

3.1. Characterization of RAM-MIP/RAM-NIP

After MIP synthesis, the morphology of the polymer was performed by SEM (Fig. 2). It can be seen from the images that the synthesized polymers with nodules size 2–3 μm were clustered. The large pore volumes allow biomolecules pass through rapidly while the small analyte molecules are retained in the polymers. Furthermore the roughly surface of the polymers might provide a larger surface area. More special imprinting could exist in the surface to rebind the template.

The TGA over the temperature range of 30–800 °C was performed with heating rate of 10 °C min^{-1} in nitrogen atmosphere [24,28]. The TGA curves of the RAM-MIP and RAM-NIP given in Fig. 3 showed a two-stage decomposition pattern for the two polymers. At temperatures from 30 to 100 °C, their weight loss were mainly due to the loss of water, and the weight loss increased rapidly from 300 to 420 °C, which can be attributed to the loss of the thermal decomposition of the polymer. Beyond 500 °C, the entire polymers were completely decomposed. The fastest weight loss occurred from 306 °C to 362 °C and 335 °C to 420 °C for the RAM-MIP and RAM-NIP absorbents, respectively. The difference of TGA curves hints that the interaction between monomer and template in the prepolymerization effects the thermal decomposition of the polymer.

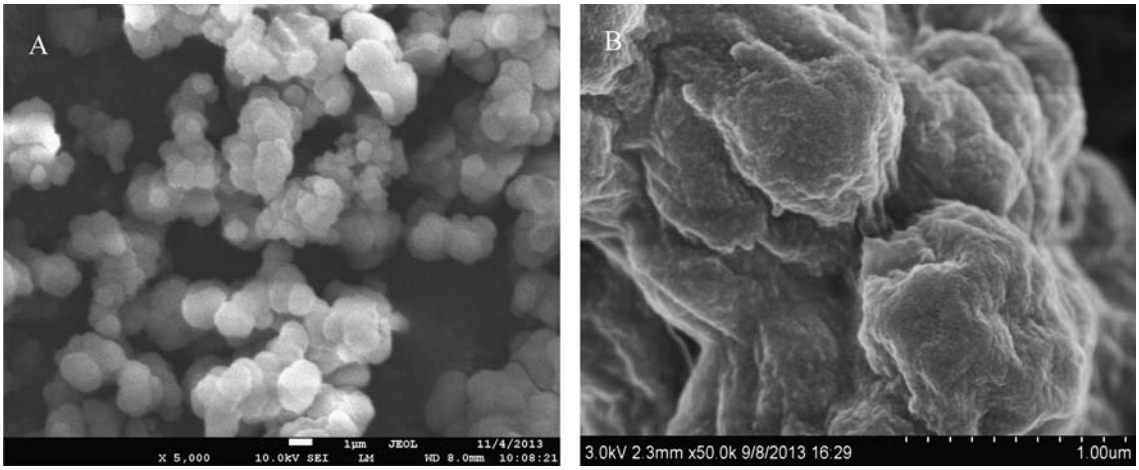

Fig. 2. Scanning electron micrographs (SEM) of RAM-MIP in the best mass ratio between monomers and porogen: (A) 3000 × ; (B) 20,000 × the polymers were synthesized by non-covalent precipitation polymerization.

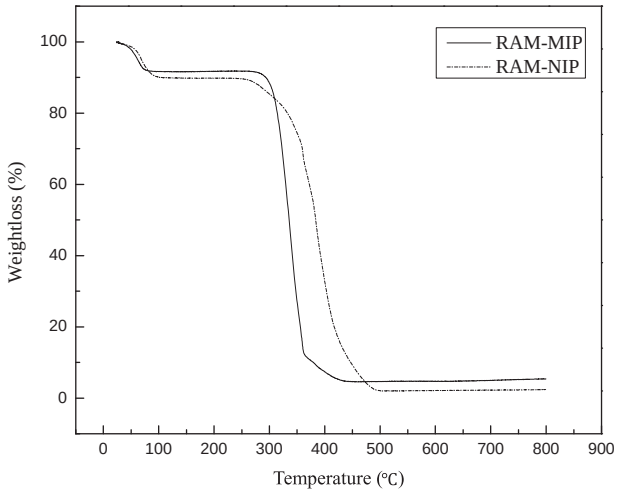

Fig. 3. TGA curves of the RAM-MIP (A) and RAM-NIP (B).

3.2. Water dispersion and protein binding experiments

The above-obtained RAM-MIP and MIP polymers were characterized with water dispersion experiments [29]. There were much more sediment for the MIP in water in comparison with RAM-MIP. The RAM-MIP with GMA insertion has been proven to be highly efficient for improving the dispersion stability of the materials in water. Therefore, the particles with GMA insertion show enhanced dispersion stability in water at ambient temperature.

To prove the usefulness of the hydrophilic materials, non-specific hydrophobic adsorption of protein on the different matrices were estimated by injecting 0.5 mL standard BSA solution (1.2 mg mL^{-1}) on SPE columns packed with MIP, RAM-MIP and the corresponding non-imprinted particles. Experimental data confirmed that the accumulative adsorption of protein for the four polymers (MIP, NIP, RAM-MIP and RAM-NIP) were 60.6%, 61.7%, 6.5% and 6.3%, respectively. According to the results, RAM-MIP and RAM-NIP showed the lower absorption of protein. The results agreed with the expected order of decreasing hydrophobicity where polymers of RAM series possessed the most hydrophilic character and could be less susceptible to fouling by proteins.

During the RAM-MISPE procedure, RAM-MIP can prevent large molecules, such as proteins, from entering small pores. On the other hand, the target molecules and the interfering of small molecules are enriched into the polymers. Then only the target analytes are effectively and selectively adsorbed on the imprinted cavity of the RAM-MIP owing to specific imprinting interaction.

3.3. Batch adsorption experiments

3.3.1. Effects of pH

Generally, analytes are adsorbed by the sorbent in molecular form. An analyte as ionic or molecular form bases on the pH of the sample solution. So the effect of pH on the rebinding efficiency of 2-ME was investigated by varying the solution pH from 4.0 to 8.0 [30]. Several batch experiments were performed by equilibrating 20 mg of the RAM-MIP and RAM-NIP particles with 5 mL of solutions containing of 2-ME (30 μg mL^{-1}) in the desired pH range. According to the result in Fig. 4, the extraction amount of 2-ME reached the largest value at pH 7. 2-ME appeared in the ionic state at low or high pH values. A decrease of the adsorption amount at higher acidic or basic concentration might result from the increasing ionic strength of the analyte, which was not advantageous for extraction. In either of the cases hydrogen binding formation between 2-ME and polymer is impossible [31]. Therefore, the optimum pH value of the sample solution was 7 throughout the experiments.

3.3.2. Sorption kinetics and sorption isotherms

The imprinting effect was evaluated by performing binding experiments as reported elsewhere [32]. For equilibrium binding assays, fixed amounts of imprinted polymers were incubated with different concentrations of 2-ME standard solution which was prepared in methanol–water (5/95, v/v). The static adsorption data (Fig. 4B) showed the binding capacity of imprinted polymer increased with the increasing of the initial concentration of 2-ME, and displayed a higher affinity for template molecule than non-imprinted polymer. The tests clearly proved the specificity of the interaction between the template and the functional groups on the polymeric particles. As Fig. 4C depicted, RAM-MIP has a stronger memory function and higher adsorption capacity for 2-ME than RAM-NIP. The adsorption equilibrium reached within 60 min. It is obvious from the fact that a large number of vacant surface imprinted sites are available for adsorption during the initial stage. After elapse of time, the deeply vacant imprinted sites are occupied gradually. The adsorbed amount of the RAM-MIP was 1.49 times than that of the RAM-NIP under equilibrium adsorption.

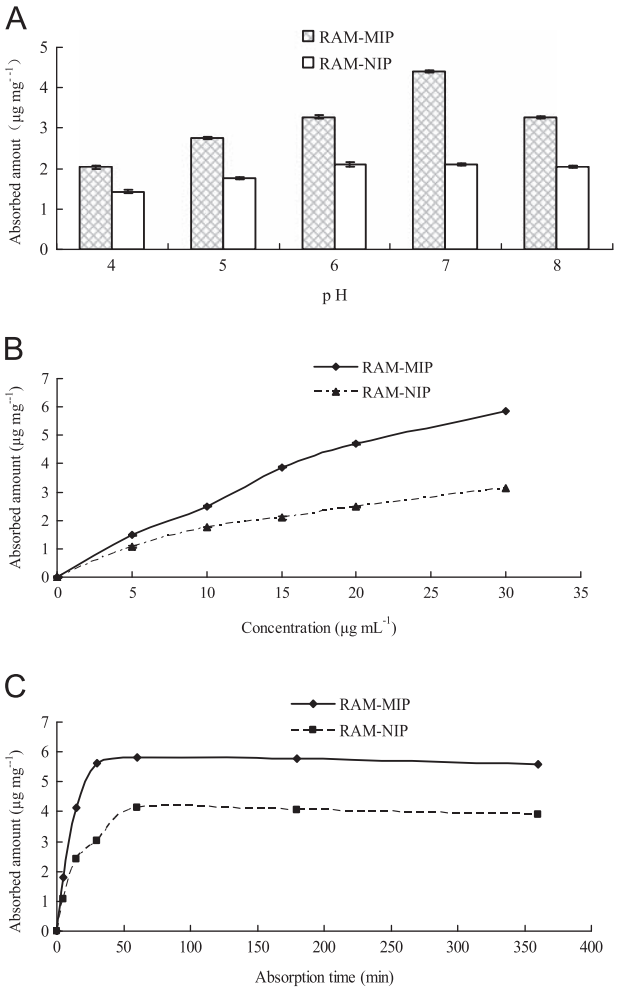

Fig. 4. Adsorption capacities of the RAM-MIP and RAM-NIP particles for 2-ME. (A) Adsorption amounts obtained using RAM-MIP and RAM-NIP at different pH. (B) Adsorption dynamics of RAM-MIP and RAM-NIP for 30 μg mL^{-1} 2-ME. (C) The adsorption isotherms of RAM-MIP and RAM-NIP to 2-ME in water–methanol (7:3, v/v) solutions.

3.4. Retention properties of various compounds on RAM-MIP polymers

The selective binding of drugs (2-ME, estradiol, hydrocortisone acetate, glibenclamide, 10 μg mL^{-1}) to the RAM-MIP was carried out off-line using RAM-MIP cartridges that were manually packed. Methanol was used for conditioning and washing, and it was chosen due to its miscibility with water and versatility in laboratory work. Solution of acetic acid in methanol (1:9, v/v) was used to desorb the selectively bound analytes from the imprinted polymers. Every compound was percolated through the RAM-MIP and RAM-NIP cartridges. The cartridges were washed with increasing amounts of methanol in water. The washing recoveries of analytes in RAM-MISPE were investigated in comparison with RAM-NISPE. The samples were detected three times by HPLC analyses.

Fig. 5 shows that there are significant differences between the bindings of 2-ME/estradiol to the RAM-MIP (Fig. 5A) and that of 2-ME/estradiol to the RAM-NIP (Fig. 5B). The analyte is released from the reference polymer (NIP) during the loading and washing steps. In contrast, RAM-MIP shows higher affinity towards the target analytes (2-ME and estradiol) than both hydrocortisone acetate and glibenclamide. The relative selectivity was observed when 30% methanol was used.

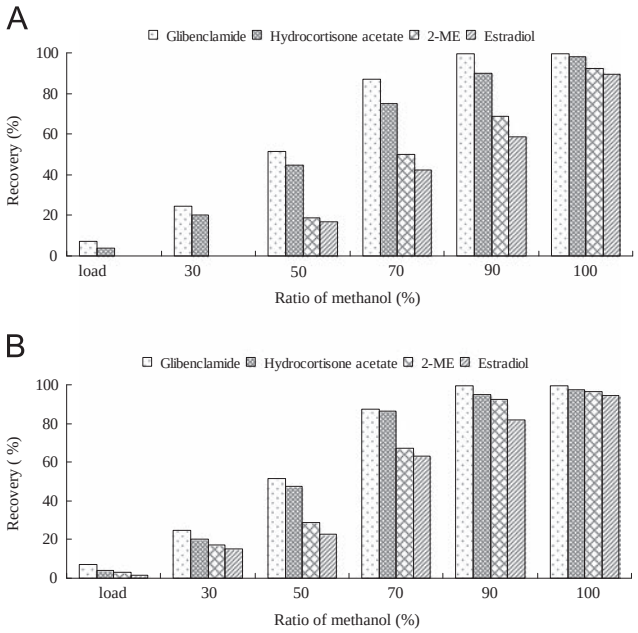

Fig. 5. The recoveries profile of analytes from RAM-MISPE (A) and RAM-NISPE (B) cartridges. 0.5 mL of samples (10 μg mL^{-1}) in water–methanol (7:3, v/v) was loaded. Methanol solution (including different amount of water) was used as the washing solvent. Wash volumes: 2 mL.

The main underlying mechanism [33,34] is attributed to formation of a 3D shape selective cavity that is complementary to their molecular skeleton. Therefore, the molecular size and shape might serve as main factors for specific recognition, together with ionic interaction, hydrophobic effect and hydrogen bond. It can be seen (Fig. 5A) that the RAM-MISPE shows better affinity for estradiol and 2-ME than the RAM-NISPE (Fig. 5B) during the loading step, and that analytes can be eluted subsequently by solution of methanol–acetic acid (9:1, v/v). The similar retention between 2-ME and estradiol was attributed to their high similarity in molecular size, shape and functional groups (–OH). The different retention between hydrocortisone acetate and template may due to the same molecular skeleton but the different functional groups. Obviously, the retentions of glibenclamide and hydrocortisone acetate were based on the non-special adsorptions so as to cause no obvious difference in the recoveries between two cartridges.

3.5. Optimization of RAM-MISPE procedure

At first, cartridges were conditioned. Samples were loaded on the MIP and NIP cartridges separately, followed by the washing solvent. The elute procedure was assessed for obtaining maximum recovery of the analytes using various solvents including acetone, acetonitrile, methanol and methanol/acetic acid. For the MISPE process, 2-ME solubilized in methanol/water (5/95, v/v) and blank plasma spiked with 2-ME working standard solutions were loaded onto the MISPE cartridges at a migration flow rate of 0.2 mL min^{-1}. 8 mL of methanol/water (5/95, v/v) followed by 3 mL of methanol/water (30/70, v/v) were employed for washing the cartridges, respectively. The analytes were eluted with different solvents (acetone, methanol, acetonitrile, methanol–acetic acid (9/1, v/v). The results depicted that during the washing step the 2-ME remained on the polymers and most of the drug was released from the polymers only in the elution step. The recovery was the best when mixture solution of methanol–acetic acid (9/1, v/v) was used as eluent. Indeed, methanol and acetic acid are polar solvents able to break hydrogen bonds between

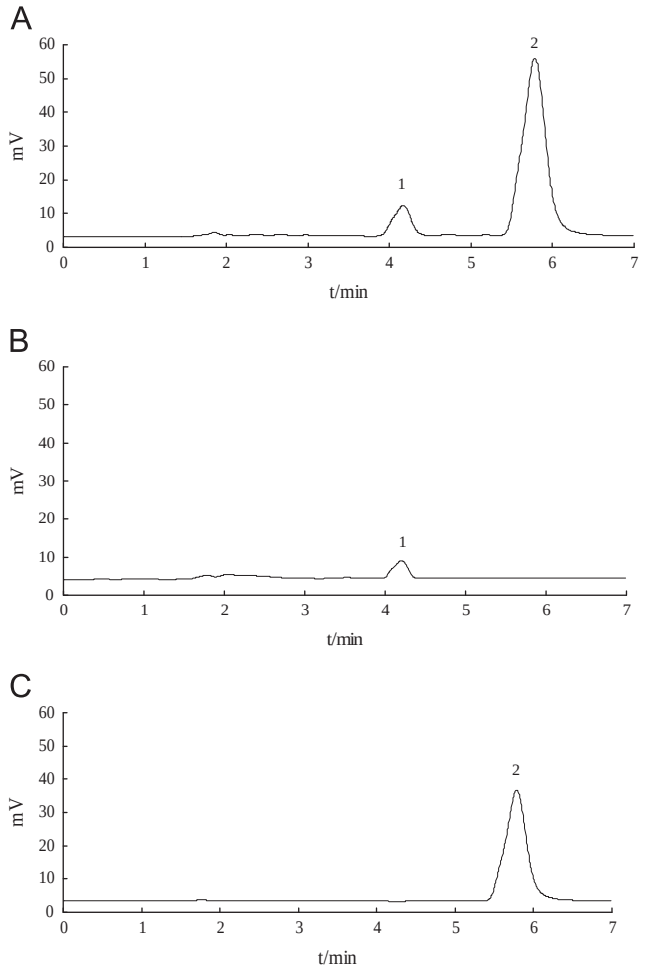

Fig. 6. The chromatograms of the samples pretreated by RAM-MISPE. (A) 2-ME plasma sample (10 μg mL^{-1}), (B) the blank plasma and (C) 2-ME solution. Peak identification: (1) estradiol and (2) 2-ME.

functional groups of 2-ME and carboxyl groups present in RAM-MIP cavities. The recovery of 2-ME methanol–water solution was 95.91% while the recovery of 2-ME spiked in plasma was 75.8%. This suggests that the presence of endogenous plasma components affects the total relative recovery of 2-ME, probably as a result of the drug binding to proteins [11].

3.6. Validation of analytical method

3.6.1. Specificity

Specificity was examined by analyzing blank plasma from three different available pools used for the preparation of matrix calibrators and quality control samples. The typical HPLC chromatograms of the blank and spiked samples are shown in Fig. 6. As can be seen, the small endogenous peaks detected in blank plasma did not interfere with 2-ME.

3.6.2. Calibration curve linearity

Blank plasma samples were spiked with 20 μL 2-ME working standard solutions (1.5–500 μg mL^{-1}) in order to generate concentrations of the 2-ME ranging from 0.06 to 20 μg mL^{-1}. Under optimized conditions, calibration curves were obtained by the RAM-MISPE protocol. The results showed a good linearity range of 0.06–20 μg mL^{-1}. The calibration curve was constructed by plotting the peak areas (y) versus the concentrations (x) of 2-ME obtained from HPLC analysis. The regression equation was expressed as $y=414.58x+4.97$ with correlation coefficients (r) of 0.9991.

3.6.3. Precision and accuracy

To assess the accuracy and precision of the method, quality control (QC) samples were spiked at concentrations of 0.16, 2.00 and 16.00 μg mL^{-1} for 2-ME (Table 1). The intra-assay precision was evaluated by five repeated injections of each QC samples and RSD were less than 10%. Inter-day RSDs of these samples were in the range of 3.5–11.9%. The results demonstrated that RAM-MISPE had acceptable recovery and precision, and could clean up and enrich 2-ME in the plasma samples.

3.6.4. Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were determined based on the signal to noise (S/N) ratio of 3 and 10, respectively. The LOD and LOQ for 2-ME standard were 0.02 and 0.06 μg mL^{-1}, respectively. Therefore, the obtained LOD and LOQ were satisfactory and allow the determination of 2-ME.

3.6.5. Reusability

The reusability of the RAM-MISPE was evaluated by comparing the adsorption of 2-ME in plasma samples. According to the experiments, 2-ME was extracted with methanol/acetic acid (9/1, v/v) overnight after use, and then the recoveries of 2-ME were obtained by RAM-MISPE-HPLC. The results are shown in Fig. 7. The RAM-MISPE could be repeatedly used nine times and it was washed with 2 mL methanol and 2 mL deionized water in turn, and then dried in vacuum each time before reusing. So the MIP is reusable, inexpensive, easy to synthesize, mechanically resistant, and stable in solvents.

3.6.6. Robustness

The robustness of a method is an estimation of its capacity to remain unaffected when small variations are deliberately introduced in the analytical parameters, an indication of the method reliability and the influence of each analytical parameter during routine operation can be achieved by this way. The robustness of the method was evaluated by analyzing data after checking seven variables according to Juan's robustness test [35]. These included the sorbent mass of SPE cartridges, flow-rate of SPE, percentage of methanol in washing solvent, volume of wash solvent, percentage

Table 1
Average recoveries (R) and relative standard deviations for the determination of 2-ME in rat plasma ($n=5$).

Analyte	Added (μg mL^{-1})	Found (μg mL^{-1})	$R \pm$ SD (%)	Inter-day RSD (%)	Intra-day RSD (%)
1	0.16	0.15 ± 0.01	99.10 ± 0.08	11.9	7.8
2	2.00	2.02 ± 0.09	101.00 ± 0.05	9.8	9.3
3	16.00	16.16 ± 0.18	100.98 ± 0.01	3.5	2.9

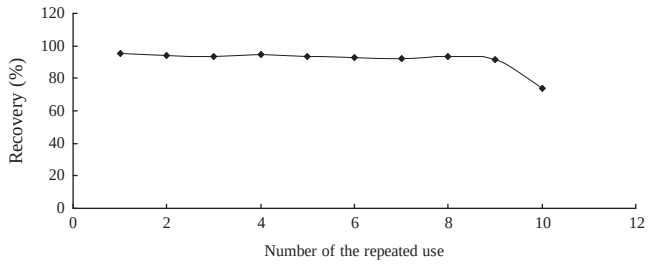

Fig. 7. Reusability of RAM-MISPE. Spiked concentration, 2 μg mL^{-1}.

Table 2
The robustness results of the method.

Factors	Sorbent mass (mg)	Flow rate (mL min^{-1})	Percent of methanol (%)	Volume of wash (mL)	Acetic acid for elution (%)	Eluent volume (mL)	Evaporation temperature (°C)
Nominal value	30	0.20	30	3.0	10	3.0	40
High value (+)	32	0.22	32	3.2	11	3.2	43
Low value (−)	28	0.18	28	2.8	9	2.8	37
Experiment 1	+	+	+	+	+	+	+
Experiment 2	+	+	−	+	−	−	−
Experiment 3	+	−	+	−	+	−	−
Experiment 4	+	−	−	−	−	+	+
Experiment 5	−	+	+	−	−	+	−
Experiment 6	−	+	−	−	+	−	+
Experiment 7	−	−	+	+	−	−	+
Experiment 8	−	−	−	+	+	+	−
Absolute difference	121	43	91	55	63	78	29
$s \times \sqrt{2}$	247						

Table 3
Determinations of 2-ME in three rat plasma samples by RAM-MISPE-HPLC and C_{18}-SPE-HPLC (n=3).

Sample (no.)	Content (μg mL^{-1})	Added (μg mL^{-1})	Found (μg mL^{-1})	Recovery (%)	RSD (%)	C_{18}-SPE (%)
1	2.03	0.16	2.21	101.0	3.52	93.1
2	2.85	2.00	4.74	97.7	3.18	94.7
3	2.51	16.00	18.21	98.4	3.75	97.3

of acetic acid in elution solvent, volume of elution solvent and evaporation temperature.

The robustness test involves eight experiments in which the nominal values of the analytical variables (factors) were slightly modified. Each experiment has a specified set of factor levels (see Table 2, where the nominal, high and low values are shown). The peak areas of the analytes in each experiment were recorded.

To evaluate the effect of each variable for each analyte, the mean of the four peak areas corresponding to low levels was subtracted from the mean of the four peak areas obtained at high levels. Then, the absolute difference value was compared with the standard deviation (s) of the eight results according to Eq. (2), following the criterion of reference [36].

$$\left|\overline{\text{Peak area}}_{\text{high value}} - \overline{\text{Peak area}}_{\text{low value}}\right| > s \times \sqrt{2} \quad (2)$$

the variable is significant.

If the value of the difference is higher than the standard deviation multiplied by square root of two, the variable has a significant effect and the method is sensitive to changes in the variable concerned. Table 2 also shows the absolute difference values and the standard deviation criterion. According to the above-mentioned criterion there were not significant effects for the studied variables when slight modifications were introduced. So the procedure can be considered as robust.

3.7. Extraction of 2-ME from rat plasma

In this study, three rats were intravenous administered 40 mg kg^{-1} of 2-ME. Blood was obtained from the orbital vein at 10 min after administration of the drug. The samples were cleaned up by RAM-MISPE and C_{18} procedures. As a control experiment, extraction on RAM-NISPE was investigated in similar conditions. The samples were then used HPLC for the determination of

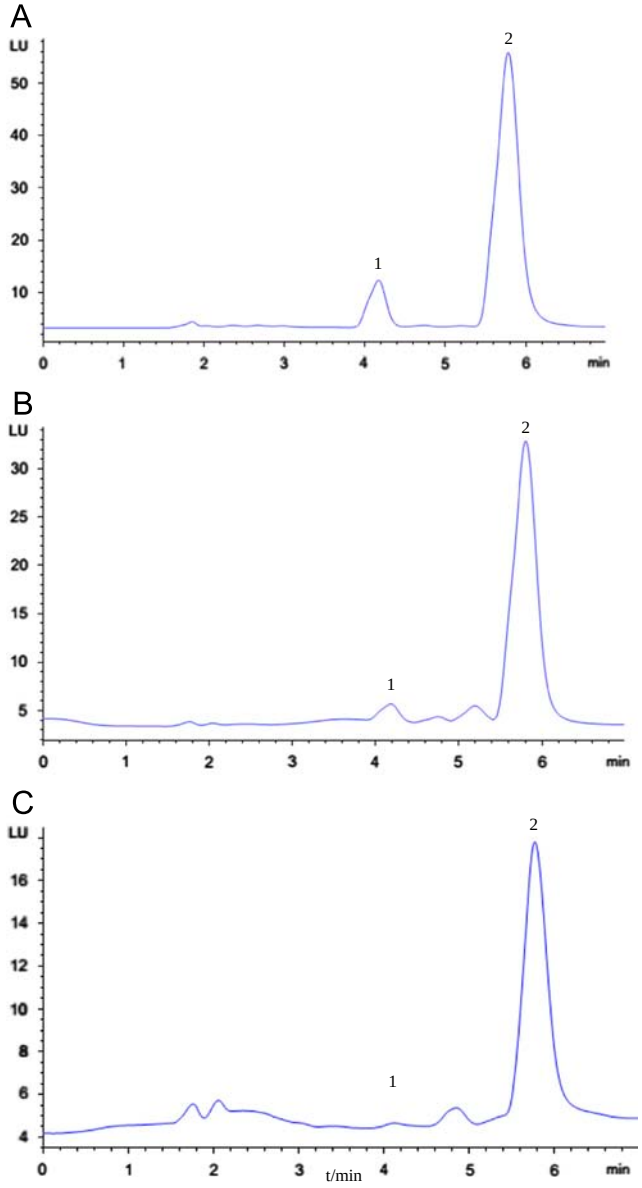

Fig. 8. The chromatograms of the plasma samples pretreated by (A) RAM-MISPE, (B) C_{18} SPE and (C) RAM-NISPE. Peak identification: (1) estradiol and (2) 2-ME.

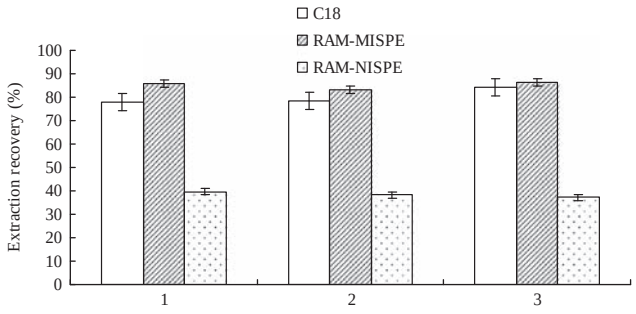

Fig. 9. The extraction recovery of the plasma samples pretreated by RAM-MISPE, C_{18} SPE and RAM-NISPE ($n=3$).

contents and recoveries. Results are summarized in Table 3. It was found that the spiked recoveries were acceptable.

The chromatogram confirmed that selective clean-up of MISPE increased sensitivity and accuracy of HPLC analysis (Fig. 8). MISPE gave a better baseline and a better HPLC separation efficiency (Fig. 8A), compared with plasma samples after C_{18} SPE (Fig. 8B), or NIP-SPE (Fig. 8C) extraction. The chromatogram of C_{18} extracted samples showed more complex matrices peaks due to some trace interfering components that were also preconcentrated in the samples. Comparing with C_{18} SPE procedure the RAM-MIP has less interfering peaks. RAM-MISPE protocols afforded both good selectivity and acceptable extraction recoveries ($85.14 \pm 0.66\%$). These data indicated that the MISPE with selective binding capacity is more suitable to separate and enrich trace analyte from complex matrix than C_{18} SPE. Off-line MISPE combined with HPLC is a reliable method to determine trace 2-ME in plasma samples with high accuracy and repeatability. The results (Fig. 9) showed that RAM-NISPE led to a low extraction recovery, generally less than 40%. The extraction recovery was $80.13 \pm 3.49\%$ for C_{18} SPE. This was because most of the analytes were removed by washing solutions due to its low affinity to 2-ME.

4. Conclusions

In this paper, restricted access materials combined to molecularly imprinted polymers were synthesized via a non-covalent molecular imprinting approach in acetonitrile for selective extraction of 2-ME from plasma samples. Molecular binding capability, selectivity, protein binding property and analytical applications of the RAM-MIP were evaluated. The presented study demonstrates that the application of RAM-MIP as SPE sorbent is sufficient and reproducible in the pretreatment of biological fluids. Comparing with the traditional sample pre-treatment [37], the plasma samples were directly loaded onto the cartridges without deproteinization of the biological fluids. The development of a fast, accurate and selective analytical method in this study provides a strategy for development of pretreatment and related analytical means for further drug detection in biological samples. Furthermore, the results obtained are the basis for further studies on the application of RAM-MIP as sorbents for online-SPE in combination with HPLC.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.05.005.

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