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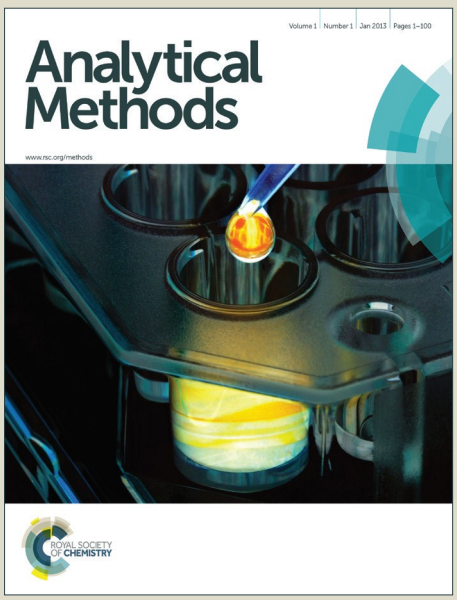

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Selective micro solid-phase extraction of epinephrine, norepinephrine and dopamine from human urine and plasma by aminophenylboronic acid covalently immobilized on magnetic nanoparticles followed by high-performance liquid chromatography-fluorescence detection

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Abstract

Aminophenylboronic acid–functionalized magnetic nanoparticles were synthesized with a facile method for the selective micro solid-phase extraction of norepinephrine, epinephrine and dopamine. After extraction, HPLC with fluorescence detection was used for the determination of the analytes in human urine and plasma. The effects of experimental parameters such as sorbent amount, sample pH, extraction and desorption conditions on the extraction efficiency of the sorbent were investigated. The selectivity of the sorbent was also evaluated. The linearity range of the method was 0.04-10 ng mL^{-1} for norepinephrine and epinephrine, and 0.06-25 ng mL^{-1} for dopamine. The recovery of the analytes was in the range of 96.8-97.5% and 86.3-88.1% for urine and plasma samples, respectively. Also, the relative standard deviations were in the range of 3.0-4.5 and 4.0-5.0% for urine and plasma, respectively. The limits of detection were 0.01, 0.01 and 0.02 ng mL^{-1} for norepinephrine, epinephrine and dopamine, respectively.

Keywords: *micro solid-phase extraction, magnetic nanoparticles, aminophenylboronic acid, catecholamines, biogenic amines, plasma, urine*

1. Introduction

Catecholamines are neurotransmitters that have many important functions in the brain and body of human and all animals. Several important diseases of the nervous system are associated with the malfunction of catecholamines system, especially dopamine, norepinephrine and epinephrine. Many diseases like Parkinson, schizophrenia and hypertension,[1] stress, neuromuscular disorders and various mental diseases,[2] Alzheimer and various cancers,[3] severe head trauma[4] and heart disease[5] are caused by the abnormal concentration of these neurotransmitters. These biogenic amines play important roles in motor control, cognition, lactation, sexual gratification, nausea, immune system, kidneys and pancreas, and many other important functions inside and outside the nervous system. Because of these important and crucial functions on body and brain, the quantification of catecholamines in biological fluids and tissues is an ongoing interest and a variety of methods have been developed to extract and detect biogenic amines.

Due to the very low concentration of catecholamines in plasma (sub-ppb levels), a very sensitive method is needed to detect these compounds. Various analytical methods have been developed to determine catecholamines in blood samples. These include gas chromatography-mass spectrometry,[6] radioactive enzymatic technique,[7] chemiluminescence,[8,9] liquid chromatography using electrochemical detection (ECD),[10-12] mass spectrometry,[13] fluorescence detection after post-column[14] and pre-column[15] derivatization, and native fluorescent detection (FLD).[16] For GC analysis, catecholamines must be derivatized before analysis. Despite high sensitivity, it is time consuming and laborious. It may lead to poor reproducibility too. HPLC with electrochemical and fluorescence detections can be regarded as the most widely used techniques for the determination of catecholamines. Electrochemical detectors are not suitable with gradient elution and need frequent attention.[14,17] Also, interfering peaks from many other oxidizable

compounds in plasma have usually been seen in the chromatogram.[18] Similarly, in HPLC-FLD, many fluorescent compounds may appear as interfering peaks in the chromatogram of biological samples.[19] To solve the problem, a hyphenated LC technique such as LC-MS/MS and/or a selective sample preparation method is often required to eliminate the interferences and obtain accurate results.

There are many papers describing different pretreatment and extraction methods for the determination of catecholamines in complex biological matrixes. Liquid-liquid extraction,[20] on-line and off-line solid-phase extraction utilizing C_{18} adsorbent,[21] Sephadex G10,[22] cation-exchange resin[23] and alumina[24] have been applied as non-selective sample preparation techniques. Selective extraction of catecholamines using boronate gels[25,26] and immobilized boronic acid on silica adsorbent[27] has also been reported.

Today, with great advances in nanotechnology, the use of nanoparticles in separation sciences is an ongoing trend. The use of magnetic nanoparticles (MNPs) as a new sorbent in solid-phase extraction procedures has recently attracted a great deal of attention because of its advantages over traditional sorbents used in the solid-phase extraction. Fast and efficient extraction, high enrichment factor, high surface area, modifiable surface, simple isolation from matrix, biocompatibility and often, the reusability of these fantastic sorbents are the great advantages over other sorbents.

Very recently, the use of MNPs coated with 3-aminophenylboronic acid (3-APBA) followed by HPLC-ECD for the determination of catecholamines in urine has been reported.[28] Considering the synthesis process, it is obvious that 3-APBA has been adsorbed on the nanoparticles through acid-base interactions. The efficiency of the method (including extraction and detection steps) was not high enough to reach very low detection limits. Therefore, the method was not suitable for the quantification of catecholamines in blood samples.

In this study, MNPs were coated with a silica layer through a sol/gel process using tetraethoxysilane. Then, for the synthesis of the selective sorbent, 3-aminophenylboronic acid was covalently immobilized on the surface of silica coated MNPs previously modified with 3-aminopropyltriethoxysilane and glutaraldehyde. The sorbent was used for the selective micro solid-phase extraction (MSPE) of three catecholamines (norepinephrine, epinephrine and dopamine) from plasma and urine samples. The selectivity of the analytes on the sorbent was compared to that of uncoated Fe_3O_4 MNPs. HPLC with fluorescence detection was used for the quantification of the analytes in the extracted samples.

2. Experimental

2.1. Chemicals and standards

Ferric chloride hexahydrate ($FeCl_3.6H_2O$, >99%), ferrous chloride tetrahydrate ($FeCl_2.4H_2O$, >99%), ammonia solution (25% w/w), sodium hydroxide (>99%), hydrochloric acid fuming (37% w/w), tetraethoxysilane (TEOS, >98%), trifluoroacetic acid (TFA ≥99%), sodium cyanoborohydride ($NaCNBH_3$), methanol (HPLC grade, ≥99), acetonitrile (HPLC grade, ≥99) and glutaraldehyde (GA, 25% w/w) were obtained from Merck (Darmstadt, Germany). Sodium methasilicate (Na_2SiO_3) and 3-APBA hemisulphate were obtained from Sigma-Aldrich (Buchs, Switzerland). Anhydrous ethanol (>99.6%) was purchased from Bidestan Company (Ghazvine, Iran). 3-aminopropyltriethoxysilane (APTES, 98%) was obtained from Fluka (Buchs, Switzerland). Epinephrine, dopamine hydrochloride and norepinephrine bitartrate were obtained from Caspian Tamin Pharmaceutical Company (Tehran, Iran). 4-Aminophenol, 1,4-dihydroxybenzene, 1,3-dihydroxybenzene, 1,2-dihydroxybenzene and 3.5-dihydroxytoluene used for study the selectivity of the sorbent were also purchased from Merck.

Pure water was prepared by OES water purification system (Oklahoma, USA).

The stock standard solution of each analyte (1000 mg L^{-1}) was prepared in water (acidified with a few drops of HCl) and stored at 4 °C in a light resistant container. A mixed standard solution of the analytes was prepared in water at 10 mg L^{-1} concentration level. Working standard solutions were prepared in water using the mixed standard solution. Due to the instability of the diluted standard solutions, they were prepared immediately before use.

2.2. Apparatus and chromatographic conditions

A HP 1090-II liquid chromatograph (now Agilent, Palo Alto, CA, USA) equipped with a HP 1046A fluorescence detector and a 7725i injection valve with a 20 µL sample loop (Rheodyne, USA) was employed. The Fourier transform infrared (FT-IR) spectra were recorded using a Jasco-FT-IR-350 (Tokyo, Japan) between 4000 and 400 cm^{-1}. Scanning electron microscopy analysis was performed on a Hitachi S-4160 field emission scanning electron microscope (Tokyo, Japan) operated at 30 kV. A compact Froilabo SW14 centrifuge (Meyzieu, France) was used for blood sample preparation. The separation of the analytes was performed using a Bischoff chromatography NC2546 C_6 column (250 mm × 4.6 mm i.d., 5 µm, Leonberg, Germany). The column was connected to a C_8 guard column (10 × 4 mm, 5 µm) supplied from Merck. Separation was performed isocratically using 0.05% aqueous TFA-methanol (97:3, v/v) solution as mobile phase. The flow rate of the mobile phase was maintained at 1 mL min^{-1} during the separation. Detection was performed by native fluorescence using excitation at 230 nm and emission at 310 nm. All separations were performed at room temperature.

For the experiments performed to test the selectivity of the sorbent a Hector-M Phenyl column (15 cm × 3 mm i.d., 3 µm, RStech Co, Daejeon, Korea) was used for the separation of the compounds. The separation was performed isocratically with 25% acetonitrile and 75% water (0.1 M H_3PO_4) at a flow rate of 0.5 mL min^{-1}. Detection was performed at 280 nm.

2.3. Synthesis procedures

2.3.1. Synthesis of Fe_3O_4@SiO_2-TEOS composite nanoparticles

0.8445 g of $FeCl_2.4H_2O$ and 2.0093 g of $FeCl_3.6H_2O$ (Fe^{3+}/Fe^{2+} molar ratio of 1.75) were dissolved in 75 mL of pure water at 85 °C. Then, 5.6 mL of ammonia solution (25% w/w) was quickly added to the resulting solution and vigorously stirred (800 rpm) under nitrogen atmosphere for 0.5 h. The resulting precipitated particles were collected with the help of a magnet (1.4 Tesla), and washed several times with water. Then, 60 mL of water containing 0.2 g of NaOH and 0.9785 g of Na_2SiO_3 was added on the MNPs. After 5 min ultrasonication, the pH of the solution was adjusted to 6 with 2 M HCl. Then, the Fe_3O_4@SiO_2 nanoparticles were washed with water and collected with the help of the magnet. In order to obtain a porous silica coating on the surface of Fe_3O_4@SiO_2 nanoparticles, a sol-gel process was used. The Fe_3O_4@SiO_2 nanoparticles were dispersed in a solution containing 5 mL of ethanol, 5 mL of water and 110 µL of ammonia solution (25%) with the help of ultrasonication (5 min). Then, 5 mL of ethanol containing 20 µL of TEOS was added dropwise to the solution under mechanical stirring. The reaction was carried out at 80 °C for 30 min. Finally, the Fe_3O_4@SiO_2-TEOS nanoparticles were washed with water and used for the next step.

2.3.2. 3-APBA covalent immobilization on Fe_3O_4@SiO_2-TEOS nanoparticles

The covalently immobilized phenylboronic acid nanoparticles were synthesized according to a previously reported procedure.[29] The Fe_3O_4@SiO_2-TEOS nanoparticles were re-dispersed in 5 mL of absolute ethanol with the help of ultrasonication. Then, 150 µL of APTES was added and the solution was stirred mechanically at 25 °C for 60 min. The amino-functionalized magnetic nanoparticles were collected by magnetic decantation and re-dispersed in 5 mL of phosphate buffer solution (100 mM) containing 1 mL of GA at pH 7.4.

The solution was stirred continuously at room temperature for 6 h. In order to enhance the stability of immobilized GA, the C=N bond formed between APTES and GA must be reduced to C–N. Therefore, 100 μL of 0.1 mg mL^{-1} $NaCNBH_3$ was added to the solution. After that, the nanoparticles were collected and re-dispersed in 5 mL of 100 mM phosphate buffer solution (containing 0.0679 g of 3-APBA and 0.02 g of $NaCNBH_3$) at pH 7.4 and gently stirred for 3 h at room temperature. At the end, the Fe_3O_4@SiO_2@APTES@GA@3-APBA nanoparticles were washed with water and dried at room temperature.

2.4. Real sample analysis

2.4.1. Urine sample

Because of the paroxysmal nature of catecholamines secretion, only 24-h urine sample should be used for analysis.[30] Urine sample (24-h sample) was collected from a 24 years old healthy male person and collected in a plastic bottle containing 20 mL of 18 wt% HCl as stabilizer and stored at -20 °C. The sample was thawed and filtered with a 0.45 μm Nylon filter (Supelco, Bellefonte, PA, USA) and diluted for extraction procedure.

2.4.2. Blood sample

Blood samples were obtained from two healthy volunteers (24 and 26 years old). About 5 mL of fresh blood was transferred to a cold centrifuge tube and immediately centrifuged at 11000 rpm for 10 min to separate plasma. In order to precipitate proteins, 2.5 mL of $HClO_4$ solution (8%, v/v) was added to plasma and immediately centrifuged at 11000 rpm for 10 min. The samples were neutralized with concentrated NaOH solution (saturated solution) before extraction.

The study was performed according to the national and institutional guidelines, and approved by the institutional committee of Isfahan University of Technology (Isfahan, Iran). The volunteers were informed about the aims of the research and signed an informed consent.

2.5. MSPE procedure

For each experiment, 5.0 mg of 3-APBA-functionalized MNPs were added into 5 mL of sample solution in a 10 mL vial. Then, 260 μL of phosphate buffer solution (1 M) at pH 9.3 was introduced into the mixture and ultrasonicated for 1.0 min. After extraction, nanoparticles were isolated from the suspension by the magnet. To remove interference species, the nanoparticles were washed using 100 μL of methanol-acetonitrile mixture (1:1) with ultrasonication for 1.0 min. After isolation of the washed nanoparticles, 50 μL of 0.2 M acetic acid (as desorption solution) was added into the vial and the analytes were eluted by the help of ultrasonication for 4.0 min. Finally, 20 μL of the solution was injected into the HPLC system for analysis.

3. Results and discussion

3.1. Synthesis and characterization of MNPs

The synthesis of 3-APBA immobilized MNPs is schematically shown in Fig. 1. To improve the chemical resistance of the sorbent during extraction, a layer of SiO_2 was coated on the Fe_3O_4 nanoparticles using Na_2SiO_3. The SiO_2 coating also helped preventing the agglomeration of Fe_3O_4 nanoparticles.[31] In order to increase the surface area of the sorbent, a sol-gel process was applied using TEOS to create a porous coating on Fe_3O_4@SiO_2. The surface of the sorbent was activated with APTES and then GA was covalently immobilized on the Fe_3O_4@SiO_2@APTES. Finally, 3-APBA, as a selective reagent for catecholamines,

was attached on the Fe_3O_4@SiO_2@APTES@GA through the chemical reaction with GA immobilized on the sorbent surface.

FT-IR spectra of Fe_3O_4, Fe_3O_4@SiO_2, Fe_3O_4@SiO_2@APTES, Fe_3O_4@SiO_2@APTES@GA and Fe_3O_4@SiO_2@APTES@GA@3-APBA nanoparticles are shown in Fig. 2. The peaks at 582 cm^{-1} and 632 cm^{-1} observed in all spectra are related to the Fe-O bond and the peak around 3440 cm^{-1} is assigned to –OH vibration on Fe_3O_4 surface. The bands at 1087 cm^{-1} and 793 cm^{-1} in the spectra b-e correspond to asymmetric and symmetric stretching of Si-O-Si. The bands at 1554 cm^{-1} and 2924 cm^{-1} in Fig. 2c are assigned to the $–NH_2$ vibration and C-H adsorption, indicating the modification of the surface with APTES. The peak at 1721 cm^{-1}, as shown in Fig. 2d is due to the C=O bond of the glutaraldehyde. The absence of the C=O band in Fig. 2e and the presence of a peak at 1338 cm^{-1} which is related to the B-O adsorption, demonstrating the successful immobilization of 3-APBA on the surface of nanoparticles.

Field emission scanning electron microscope images of the synthesized Fe_3O_4@SiO_2 (left image) and 3-APBA immobilized MNPs (right image) are shown in Fig. 3. According to the images, the particles exhibited a well-formed spherical shape. It was also observed that after the immobilization of 3-APBA on to the MNPs surface, the aggregation was decreased and a good distribution size of the sorbent was achieved.

3.2. Optimization of extraction conditions

To assess the capability of the sorbent for the selective extraction of epinephrine, dopamine and norepinephrine from biological fluids, several experimental parameters were studied. The parameters that could probably influence the performance of the extraction, including the amount of the sorbent, sample pH, extraction time, washing and desorption conditions were considered. All experiments were performed three times for the calculation of standard deviations.

3.2.1. Amount of MNPs

In order to investigate the optimum amount of nanoparticles in the MSPE procedure, amounts in the range of 2.0-8.0 mg were used. The results (Fig. S1, Electronic Supplementary Material) showed that the extraction efficiency of catecholamines was increased with enhancing the amount of nanoparticles up to 5.0 mg, and then decreased when higher amounts of the nanoparticles were used. Amounts less than 5.0 mg were not enough for the sufficient extraction of the analytes from sample solution; therefore, the extraction efficiencies were lower than the optimum point. At higher sorbent amounts, the nanoparticles could not be properly dispersed in the solution. So, a decrease in the extraction efficiency was observed for amounts higher than 5.0 mg. Based on these results, a 5.0 mg amount of Fe_3O_4@SiO_2@APTES@GA@3-APBA nanoparticles was selected as the optimum amount of the sorbent in the following experiments.

3.2.2. Sample pH

It is known that the pH of sample plays a vital role in the extraction efficiency of the method because it affects the efficiency of the complex formation between the analytes and boronic acid functional groups immobilized on the sorbent. Accordingly, the effect of sample pH in the range of 7.0-11.0 (adjusted with HCl or NaOH) on the extraction efficiency was investigated. The extraction efficiency was found to be increased form pH 7.0 to 9.0 and decreased with the further increase in sample pH (Fig. S2, Electronic Supplementary Material). As shown in Fig. 4, at acidic and neutral pHs, the cyclic ester could be hydrolyzed and dissociated.[32] The dissociated trigonal form of boronic acid could not react with the cis-diol group of the analytes. At a pH value close to pKa (9.2), complex formation could be facilitated because of the existence of boronic acid in the activated tetrahedral boronate

form.[33] However, in more alkaline solutions (pH > 9.0), SiO_2 coating of the sorbent was dissolved and the extraction efficiency of the sorbent was decreased due to the dissociation of boronic acid functional groups from the sorbent surface. Based on the results, the sample pH 9.0 was used for further experiments.

3.2.3. Extraction time

The extraction time is another important factor in MSPE that can affect the sensitivity and analysis time of the method. In order to investigate the effect of the extraction time (ultrasonication time) on the extraction efficiency, different extraction times, from 0.5 to 6.0 min, were evaluated. The maximum value of extraction efficiency was obtained after 1.0 min for all analytes (Fig S3, Electronic Supplementary Material). Longer extraction times had no significant effect on the response. Thus, an extraction time of 1.0 min was selected as the optimum extraction time for further experiments.

3.2.4. Washing condition

In order to achieve selective extraction using 3-APBA functionalized MNPs, a washing step was applied to remove nonspecific binding materials from the sorbent. To find out the best washing condition, the type of washing solution, solution volume and ultrasonication time were investigated. Seven different solutions were used to study the effect of washing solution on the extraction recovery of the analytes. After the extraction of a standard solution of the analytes at 10 ng mL^{-1}, a volume of 100 µL washing solution under 5.0 min ultrasonication was applied for washing the sorbent. The sorbent was then eluted with 50 µL of 0.2 M acetic acid. The same experiments were also performed using uncoated Fe_3O_4 MNPs. The results obtained using both sorbents are shown in Fig. 5. To simplify the figure, the sum of the peak height of the three analytes was considered as the response, and the responses were

normalized to the recovery obtained by the sorbent without the washing step. As shown, the extraction recovery obtained by Fe_3O_4@SiO_2@APTES@GA@3-APBA nanoparticles was higher than 62% in all conditions. It demonstrated that most analytes had been bound through the specific interaction with the sorbent and even common organic solvents such as methanol and acetonitrile were not able to desorb the analytes from the sorbents. On the other hand, extraction recoveries obtained by the uncoated sorbent were very low in comparison to the selective sorbent. Extraction recovery obtained by the uncoated sorbent without employing the washing step was about 40%. It showed that the nonspecific adsorption of the analytes had a relatively low contribution in the extraction process. In addition, most of the nonspecifically adsorbed analytes could be removed during the washing step. Among different solutions applied for washing the sorbent, methanol-acetonitrile mixture (1:1) had the highest capability to remove nonspecifically adsorbed compounds from the sorbent. At the same time, the extraction recovery of the analytes after washing with the methanol-acetonitrile solution was still acceptable (67%) for the quantification of the analytes by the method. Therefore, to achieve the highest selectivity, the methanol-acetonitrile mixture was selected as the washing solution.

The effect of washing solvent volume and washing time on the extraction efficiency was studied in the range of 100-1000 µL and 1.0-5.0 min, respectively. The extraction efficiencies of three catecholamines were found to be decreased by increasing the washing volume from 100 to 1000 µL (Fig. S4, Electronic Supplementary Material). Therefore a 100 µL volume of washing solvent was used for further experiments. To find out the optimum washing time, a 100 µL washing solution containing the sorbent was ultrasonicated from 0 to 5.0 min. It was observed that with increasing the washing time from 1.0 to 5.0 min, the extraction efficiency was decreased for all analytes. Therefore, a washing time of 1.0 min was selected for further experiments.

3.2.5. Elution condition

As mentioned before, the ion-pair complex between boronic acid and catecholamines could be dissociated under the acidic condition (Fig. 4). To elute the sorbent, 50 µL of acetic acid solution at four concentration levels (0.05, 0.1, 0.2 and 0.5 M) was used. In each experiment, the sorbent was ultrasonicated for 5.0 min. With enhancing acetic acid concentration from 0.05 to 0.1 M, the elution efficiency of the analytes was increased. However, there was no significant difference in the analyte signal between 0.1 and 0.5 M acetic acid. Thus, 0.2 M acetic acid solution was chosen in all following studies.

To study the effect of acetic acid solution volume on the elution efficiency, different volumes ranging from 30 to 200 µL were investigated. Based on the results (Fig. S5, Electronic Supplementary Material), 50 µL of acetic acid solution (0.2 M) had the best efficiency in desorbing the analytes from the sorbent. To find out the best ultrasonication time for analyte elution, different times from 0 to 6.0 min were studied. The elution efficiency was increased with enhancing the elution time up to 4.0 min and after that, remained almost constant. Therefore, 4.0 min ultrasonication was used for the elution of the analytes in the subsequent experiments.

3.3. Reusability of the adsorbent

In order to study the reusability of the sorbent, it was subjected to repeated extraction and elution operations cycles. After each experiment, the used sorbent was washed three times with 1 mL methanol-acetonitrile (1:1). The sorbent was then dried at room temperature. The results showed that only about 10% of the extraction efficiency was decreased after 12 extraction-elution cycles, thereby indicating that Fe_3O_4@SiO_2@APTES@GA@3-APBA nanoparticles had enough mechanical and chemical stability to be used as a sorbent. Despite

the reusability of the sorbent, due to the use of a very low amount of the sorbent in each extraction, the adsorbent was only applied once to decrease the memory effect.

3.4. Selectivity of the sorbent

To evaluate the selectivity of Fe_3O_4@SiO_2@APTES@GA@3-APBA nanoparticles, five different aromatic compounds contain one or two hydroxyl groups including 4-aminophenol, 1,4-dihydroxybenzene, 1,3-dihydroxybenzene, 1,2-dihydroxybenzene and 3.5-dihydroxytoluene were chosen. A standard aqueous solution contains the analytes at concentration level of 0.4 mg L^{-1} was used for extraction. The extraction was accomplished under the optimized condition. The extraction efficiency of the studied compounds were 86.4, 5.4, 0.5, 2.7 and 1.8 % for 1,2-dihydroxybenzene, 4-aminophenol, 1,4-dihydroxybenzene, 1,3-dihydroxybenzene, and 3.5-dihydroxytoluene, respectively. The results clearly show that the sorbent has a great selectivity toward the compounds with vicinal hydroxyl groups.

3.5. Method evaluation

Overall, the optimized MSPE conditions were as follows: 5.0 mg sorbent amount, sample pH = 9.0, 1.0 min extraction, 100 µL volume of methanol-acetonitrile (1:1) as the washing solvent, 1.0 min washing time, 50 µL of 0.2 M acetic acid as the elution solvent and 4.0 min desorption time.

To evaluate the present method, analytical parameters including dynamic range, the limit of detection, the limit of quantification (LOQ), enrichment factor, precision and recovery for the determination of epinephrine, norepinephrine and dopamine were investigated. The calibration curves for the three catecholamines were constructed by analyzing the standard solutions of catecholamines. The resulting calibration curves, as shown in Table 1, were linear over the range of 0.04-10 ng mL^{-1} for epinephrine and norepinephrine and 0.06-25 ng

mL^{-1} for dopamine. The r^2 values were 0.9974, 0.9995 and 0.9918 for epinephrine, norepinephrine and dopamine, respectively. The limits of detection, as calculated based on S/N=3, were 0.01 ng mL^{-1} for epinephrine and norepinephrine, and 0.02 ng mL^{-1} for dopamine. The limits of quantification (S/N=10) were 0.04, 0.04 and 0.06 ng mL^{-1} for epinephrine, norepinephrine and dopamine, respectively.

To evaluate the applicability of the method in real sample analysis, human urine and plasma were analyzed by the method. For recovery test, the samples were spiked with the analytes at two concentration levels. Norepinephrine and epinephrine were added to the urine samples at 20 and 40 ng mL^{-1} and the spiked concentration levels for dopamine were 50 and 70 ng mL^{-1}. For plasma samples, spiked concentration levels were 0.4 and 0.8 ng mL^{-1} for norepinephrine and epinephrine, and 1.0 and 1.4 ng mL^{-1} for dopamine. The samples were appropriately diluted before extraction.

The recoveries for urine sample were higher than 96.8% and for blood samples, they were in the range of 86.3-88.1% (Table 2). The relative standard deviations were between 3.0 and 5.0%. The results demonstrated that the 3-APBA functionalized MNPs exhibited good recoveries for the analytes. They also implied that the matrix had a negligible effect on the quantification of the analytes and the method could be applicable for the determination of the selected catecholamines in biological fluids. Fig. 6 shows the typical chromatograms of the plasma and urine samples before and after spiking with the catecholamines.

3.6. Comparison with other methods

To compare the present method with other HPLC-based techniques applied for the determination of the analytes in human urine and plasma, some analytical characteristics of the methods have been listed in Table 3. LC-MS/MS, which is a sophisticated and expensive technique, is by far the most sensitive method for the determination of catecholamine. SPE is

almost the main sample preparation technique usually combined with HPLC-ECD (amperometric and coulometric detection). Compared to these methods, the developed method has better LOQ, but the recoveries of the spiked samples are not significantly different. Also, it consumes a very low volume of an environmentally friendly solution for the elution of the sorbent (50 μL of 0.2 M acetic acid). The washing and elution volume used in the present method were found to be 10-200 and 10-40 times lower than those used by the other methods, respectively. In addition, only 5.0 mg of the sorbent was applied in the present method in comparison to the higher amount of sorbent used in SPE. Moreover, MSPE is a fast, inexpensive and simple sample preparation technique. In comparison with MSPE-HPLC-ECD [28], the present method needed a lower elution and washing solution volume. In addition, as could be observed in Table 3, the LOQs were also at sub-ppb level, making the method very suitable for the analysis of the catecholamines in blood samples.

4. Conclusions

In this work, Fe_3O_4@SiO_2@APTES@GA@3-APBA MNPs were prepared and used for the selective extraction of catecholamines from human urine and plasma followed by HPLC-FL determination. The method combined the selectivity of the coating and the advantages of MNPs. The covalently immobilized 3-APBA on MNPs showed good selectivity towards catecholamines, as compared to uncoated Fe_3O_4@SiO_2 MNPs. No organic solvent was needed to desorb the analytes from the sorbent. The relatively high analyte recovery obtained in the analysis of spiked urine and plasma samples showed that the sample matrix had no significant effect on the quantification of the analytes by the method. In comparison to other HPLC-based techniques used for the determination of catecholamines, the present method showed lower detection limits. Simplicity, short extraction time, low cost, ease of operation,

and low consumption of washing and elution solution were among the other advantages of this technique.

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Figure captions

Fig. 1. Schematic representation of the synthesis of 3-APBA immobilized magnetic nanoparticles.

Fig. 2. FT-IR spectra of (a) Fe_3O_4, (b) Fe_3O_4@SiO_2, (c) Fe_3O_4@SiO_2@APTES, (d) Fe_3O_4@SiO_2@APTES@GA and (e) Fe_3O_4@SiO_2@APTES@GA@3-APBA.

Fig. 3. Field emission scanning electron microscope images of the synthesized Fe_3O_4@SiO_2 (left image) and 3-APBA immobilized magnetic nanoparticles (right image).

Fig. 4. 3-APBA ion-pair complex formation with cis-diols.

Fig. 5. Effect of washing solvent on the extraction efficiency of catecholamines. Initial concentration of catecholamines: 10 ng mL^{-1}, sample volume: 5 mL, sample pH: 9.0, amount of sorbent: 5.0 mg, extraction time: 1.0 min, washing volume and time: 100 µL and 5.0 min respectively, elution solvent: 50 µL of 0.2 M acetic acid, elution time: 5.0 min. (*) PBS: phosphate buffer (10 mM, pH=7.4).

Fig. 6. The HPLC-FLD chromatograms of spiked and non-spiked 2-fold diluted plasma (A) and 10-fold diluted urine (B) obtained after extraction by the method. The plasma sample was spiked with norepinephrine (0.4 ng mL^{-1}), epinephrine (0.4 ng mL^{-1}) and dopamine (1.0 ng mL^{-1}). The urine sample was spiked with norepinephrine (1), epinephrine (2) and dopamine (3), at 4, 4 and 5 ng mL^{-1}, respectively.

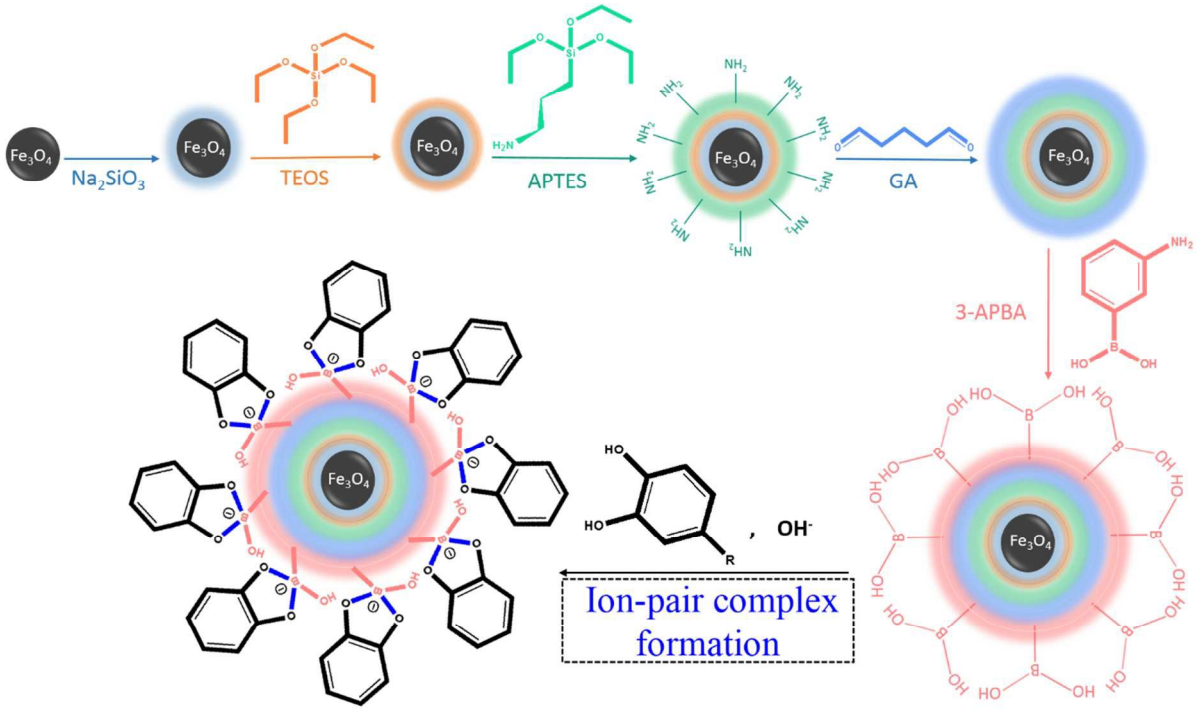


Figure 1

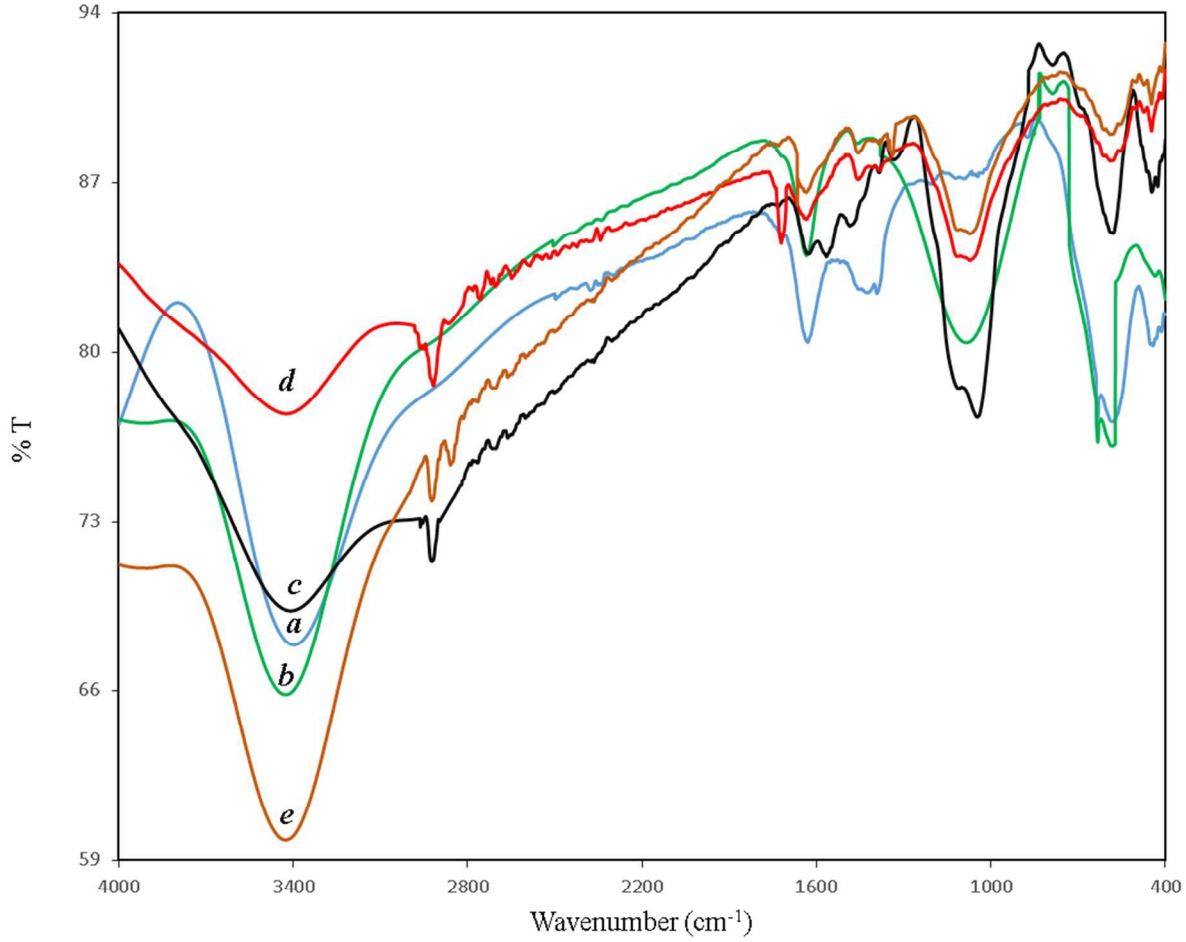


Figure 2

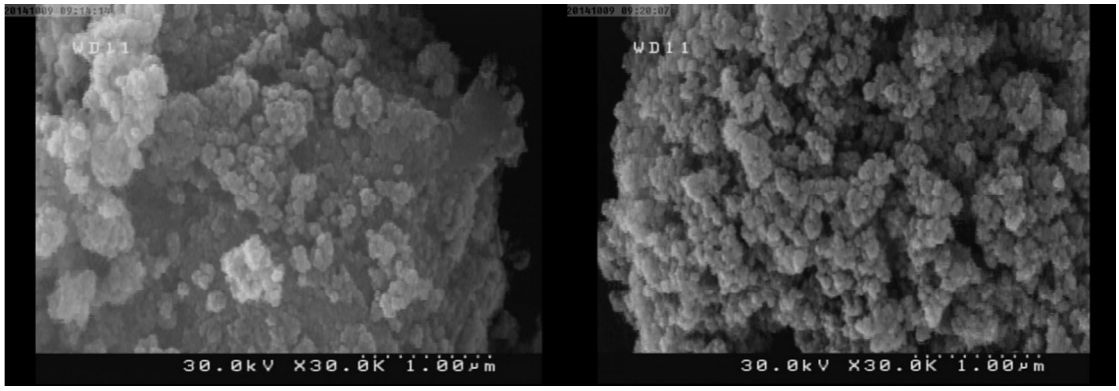


Figure 3

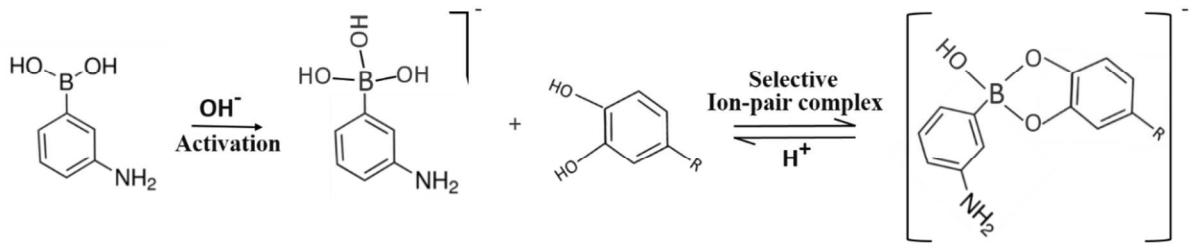


Figure 4

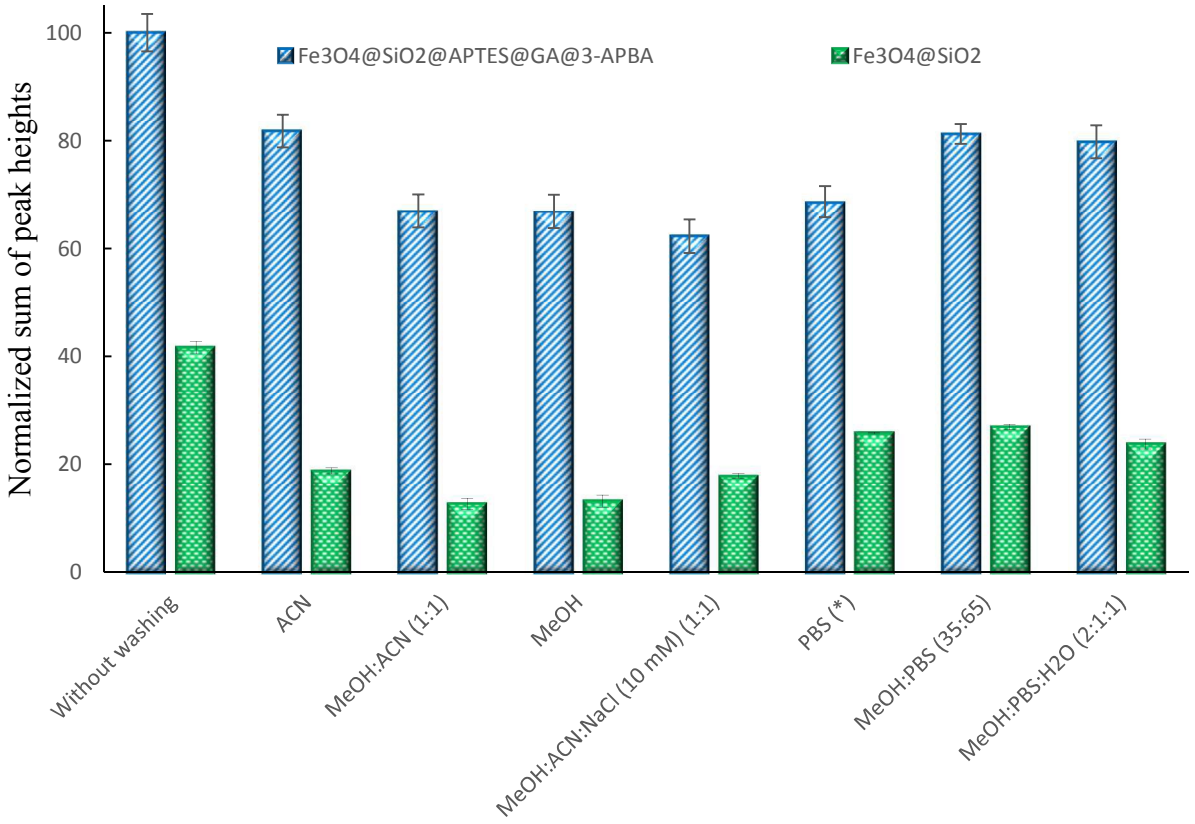


Figure 5

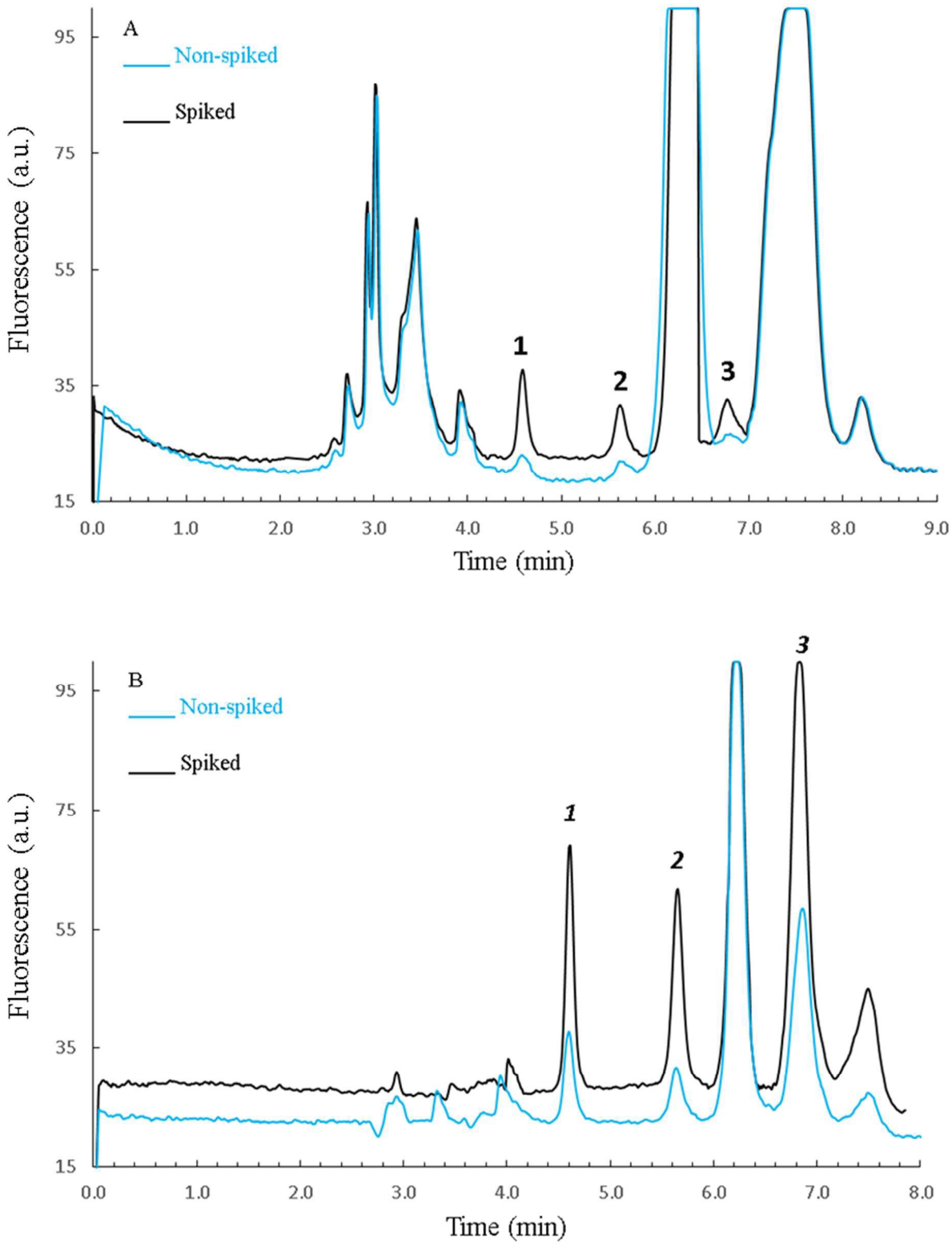


Figure 6

Table 1 Analytical performance of the method for the determination of catecholamines

Analytes	Dynamic range (ng mL^{-1})	LOD[a] (ng mL^{-1})	LOQ[b] (ng mL^{-1})	Determination coefficient (r^2)	% RSD[c] (n=3)		Enrichment factor[d]
					1 ng mL^{-1}	5 ng mL^{-1}	
Norepinephrine	0.04-10	0.01	0.04	0.9995	4.3	2.9	75
Epinephrine	0.04-10	0.01	0.04	0.9974	4.6	3.1	77
Dopamine	0.06-25	0.02	0.06	0.9918	5.0	4.3	80

[a] Limit of detection. [b] Limit of quantification. [c] Relative standard deviation. [d] Defined as the ratio of the analyte concentration obtained after the extraction/desorption process with respect to the initial sample solution.

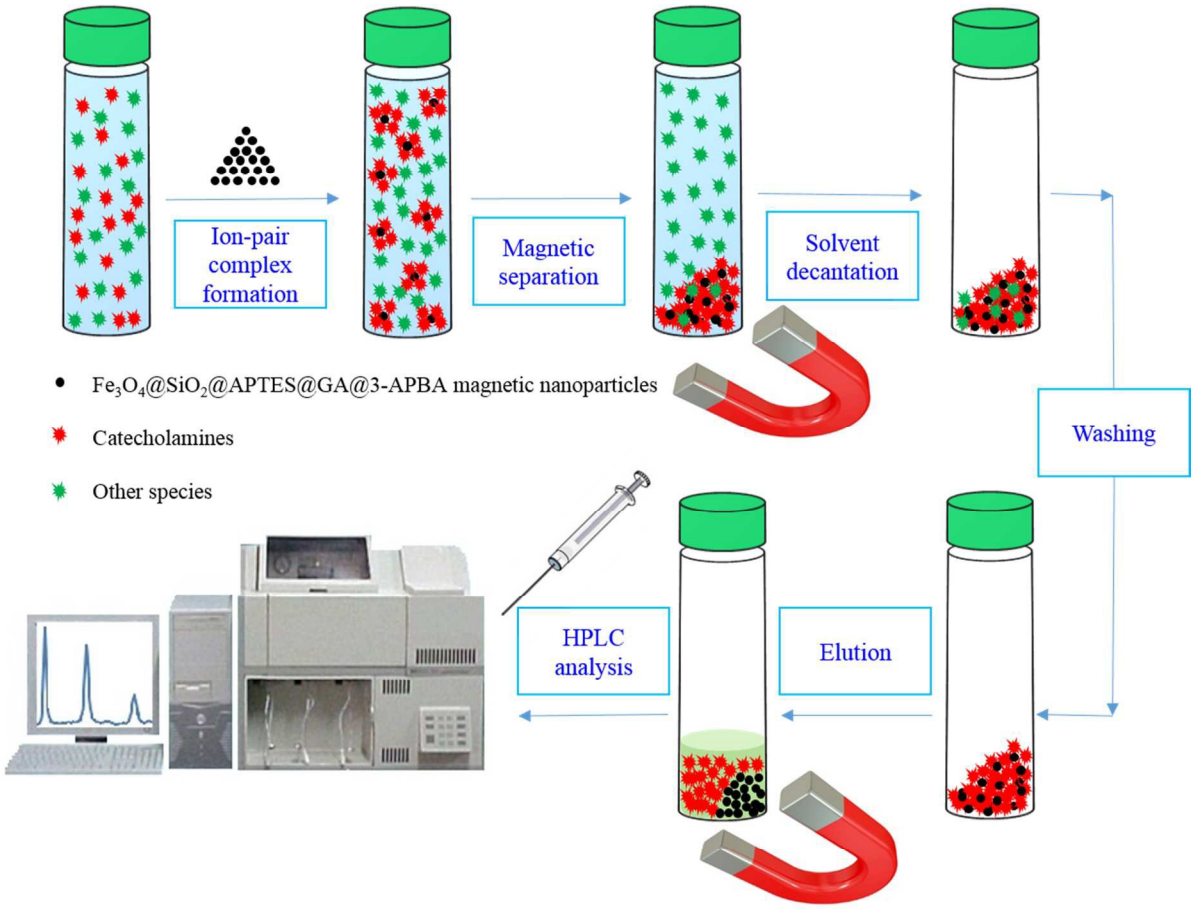
Table 2 Recovery and precision of the method for the determination of catecholamines in blood and urine samples

Sample	Analyte	Amount measured in the real sample (ng mL^{-1})	Amount added (ng mL^{-1})	Recovery (%)	RSD% (n=3)
Urine	Norepinephrine	19.70	20.00	97.5	4.1
			40.00	97.5	3.0
	Epinephrine	11.12	20.00	96.9	4.3
			40.00	97.0	3.3
	Dopamine	204.1	50.00	96.9	4.5
			70.00	96.8	4.1
Plasma I	Norepinephrine	0.48	0.40	87.5	4.8
			0.80	87.5	4.5
	Epinephrine	0.18	0.40	86.5	4.9
			0.80	86.3	4.0
	Dopamine	0.16	1.00	87.0	5.0
			1.40	87.1	4.5
Plasma II	Norepinephrine	0.65	0.40	87.1	4.7
			0.80	87.3	4.5
	Epinephrine	0.14	0.40	88.1	4.7
			0.80	88.0	4.3
	Dopamine	0.11	1.00	87.0	4.9
			1.40	87.1	4.4

Table 3 Comparison of the present method with other liquid chromatography techniques for the determination of catecholamines in biological fluids

Technique	Sample	Sample preparation	Run time (min)	Elution solvent (SPE step)	Washing volume (mL)	Elution volume (µL)	Recovery (%)	LOQ[a] (ng mL^{-1})	Reference
LC-MS/MS	plasma	SPE	6	H_2O:ACN (40:60) with 2.5% formic acid	3.2	550	66-68.1	0.005-0.02	13
LC-Coul[b]	plasma	SPE	35	THF/sodium citrate/EDTA/diethylamine/atrium dihydrogen phosphate-2-dihydrate/OSA[c]	2	2000	64.6-86.1	NR	10
LC-Coul	urine	SPE	20	1 M acetic acid	4	1500	87-91	5.41-9.80	34
LC- Amp[d]	plasma	SPE	>30	citric acid/EDTA:ACN (98:2)	2	500	63-99	0.02-0.1	11
LC- Amp	plasma	SPE	>20	citric acid/EDTA/OSA:methanol (95:5)	4	500	80-90	0.2	12
LC-ECD[e]	urine	MSPE	16	0.02 M HCl in MeOH	1	1000	92-108[f]	6.7-26.3	28
LC- FLD[g] (with Post column derivatization)	urine	SPE	15	6 M acetic acid	20	1000	98-107	NR	35
LC- CL[h]	urine	SPE	20	acetic acid	4	1500	NR[i]	0.5-1.1	9
LC-FLD (native)	urine	MSPE	8	0.2 M acetic acid	0.1	50	96.8-97.5	0.04-0.06	This work
LC- FLD (native)	plasma	MSPE	9	0.2 M acetic acid	0.1	50	86.3-88.1	0.04-0.06	

[a] Limit of quantification. [b] Coulometric detection. [c] 1-Octanesulfonic acid sodium salt monohydrate. [d] Amperometric detection. [e] Electrochemical detection. [f] Relative recovery. [g] Fluorescence detection. [h] Chemiluminescence. [i] Not reported.



Micro solid-phase extraction of catecholamines by selective aminophenylboronic acid immobilized on magnetic nanoparticles followed by high performance liquid chromatography-fluorescence detection.