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Selective Separation and Analysis of Catecholamines in Urine Based on Magnetic Solid Phase Extraction by Mercaptophenylboronic Acid Functionalized Fe_3O_4-NH_2@Au Magnetic Nanoparticles Coupled with HPLC

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Abstract: A novel magnetic solid phase extraction based on mercaptophenylboronic acid (MPBA)-functionalized Fe_3O_4-NH_2@Au nanomaterial (Fe_3O_4-NH_2@Au-MPBA) was developed for selective separation and enrichment of catecholamines (including dopamine, norepinephrine and adrenaline). Fe_3O_4-NH_2@Au-MPBA nanoparticles were achieved by self-assembly-anchoring MPBA molecules on the surface of Fe_3O_4-NH_2@Au nanocomposites, which were synthesized via a facial ultrasonic auxiliary in situ reduction process. The interaction between cis-diol from catecholamines and boronic acid was reversible and could be flexibly controlled by adjusting pH value. The catecholamines could be quickly adsorbed by Fe_3O_4-NH_2@Au-MPBA in weak alkaline solution (pH 8.0–9.0) and subsequently released in acid solution (pH 1.0–2.0). The process of adsorption and dissociation was very fast. Furthermore, the three catecholamines could be detected in urine from children by high performance liquid chromatography (HPLC) with electrochemical detector. Under optimal conditions, norepinephrine (NE), epinephrine (EP) and dopamine (DA) were separated very well from internal standard and exhibited a good linearity in the range of 2.5–500.0 ng mL^{-1}, with correlation coefficients of $r^2 > 0.9907$. Limits of detection (LOD) (signal to noise = 3) were 0.39, 0.27 and 0.60 ng mL^{-1} for NE, EP and DA, respectively. Recoveries for the spiked catecholamines were in the range of 85.4–105.2% with the relative standard deviation (RSD) < 11.5%.

Keywords: Fe_3O_4-NH_2@Au; magnetic solid phase extraction; catecholamines; HPLC; electrochemical detection; urine

1. Introduction

Catecholamines (CAs), which include norepinephrine (NE), epinephrine (EP) and dopamine (DA), are important neurotransmitters and hormones released by the adrenal glands and sympathetic nervous system [1]. Currently, it is known that catecholamines are involved in some of the most prevalent human pathologies, such as neurological disorders such as Parkinson's [2], pheochromocytoma [3] and schizophrenia [4]. Hence, monitoring the concentration of catecholamines in biological fluids including blood, urine and specific tissue has attracted considerable interest in diagnostic analysis and biological systems [5,6].

In spite of the great diversity of analytical approaches that have been developed in recent years, the detection of catecholamines in biological samples remains a hot spot in analytical fields [7,8]. High-performance liquid chromatography (HPLC) is an analytical

method routinely used for the separation and quantification of catecholamines in clinical laboratories [9,10], usually coupled with electrochemical [11], fluorescence [12] or mass spectrometry detection [13]. Among the detectors, electrochemical detectors [14,15] attract more preference due to good selectivity, highly sensitive characteristics, a lack of need for derivatization and low detection cost. However, matrix effects, extremely low concentrations and chemical instability of catecholamines in biological samples are major difficulties encountered in their analysis.

To solve these problems, optimization of the sample pretreatment process plays an important role in the enrichment and separation of targets. The common sample preparation methods for extracting catecholamines in chromatographic analysis involves liquid–liquid extraction (LLE) [16] and solid phase extraction (SPE) [17,18]. Nevertheless, LLE is labor-intensive and time-consuming [19] and encounters low selectivity and extraction yields. SPE is widely adopted for the extraction and concentration of catecholamines, possibly due to the high extraction recoveries and selectivity [20].

Magnetic solid phase extraction (MSPE) is a flexible solid phase extraction technology. The magnetic adsorbents can be recycled and reused easily, which is cost-effective and environmentally friendly. Fe_3O_4 magnetic nanoparticles (NPs), commonly used magnetic cores, are easily oxidized in air and have a tendency to aggregate [21,22]. To overcome these problems, different materials such as metals [23], metal oxides [24], mesoporous [25], polymers [26], graphene [27] and dendrimers [28] have been developed to hybridize with Fe_3O_4 NPs. In recent years, gold-coated Fe_3O_4 (Fe_3O_4@Au) magnetic NPs have drawn intense scientific and technological interest for potential applications in disease diagnosis and therapy [29], biological detection [30], catalytic application and separation science [31].

In recent years, phenylboronic acid (PBA) and its derivatives containing boronic acid functional groups have been employed for selective capture and enrichment of cis-diol molecules (carbohydrates [32], mucin [33], nucleosides and glycoconjugates) from biological samples. Owing to the ability to form a five- or six-membered boronate ester [34] via reversible covalent interactions with the position at cis-1, 2- or 1, 3-diol, they exhibit a high selectivity for targets. The formation and dissociation of boronic esters are controlled by appropriately adjusting the pH value. 4-mercaptophenylboronic acid (4-MPBA) is a thiol-containing boronic acid compound which has been used to modify gold NPs for the selective interactions with cis-diol structure on sugars [35], sialic acid, protein kinase and alpha-fetoprotein [36–39]. However, to the best of our knowledge, we have not seen this idea used for the selective separation of catecholamines.

In this report, a novel magnetic nanocomposite, 4-MPBA-functionalized Fe_3O_4-NH_2@Au (Fe_3O_4-NH_2@Au-MPBA), was prepared and used for the pretreatment of catecholamines in urine and followed by HPLC-electrochemical detection. The cis-diol structures of catecholamines were able to form ring boronate ester structures with boronic acid groups of Fe_3O_4-NH_2@Au-MPBA nanocomposite by covalent bond interaction. The morphologies and magnetization saturation values of the prepared magnetic material were explored by scanning electron microscope (SEM) and vibrating sample magnetometer (VSM). The analytical performances of the method were evaluated before determining catecholamines in urine samples.

2. Materials and Methods

2.1. Reagents and Chemicals

Dopamine (DA) and norepinephrine (NE) were purchased from Yuanye biotechnology Co., Ltd. (Shanghai, China). Epinephrine (EP) was bought from Chromadex (Irvine, CA, USA). 3,4-dihydroxybenzylamine hydrobromide (DHBA) acting as internal standard (IS) was purchased from Aladdin Reagent (Shanghai, China). 4-mercaptophenylboronic acid (MPBA) was bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Molecular structures of these compounds are shown in Figure 1.

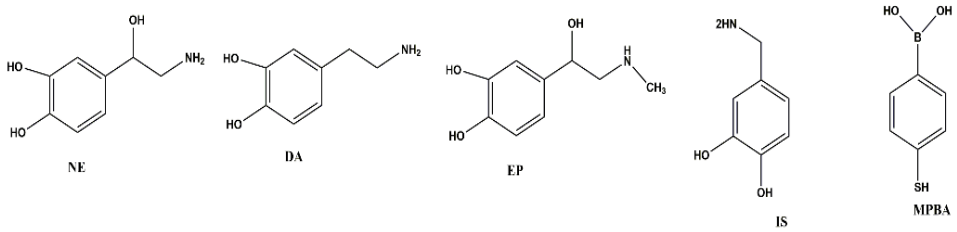

Figure 1. Molecular structures of these compounds.

Stock solutions (1 mg mL^{-1}) of NE, DA and IS were prepared by dissolving 5 mg of standard substance in 5 mL of deionized water. Stock solution (1 mg mL^{-1}) of EP was prepared by dissolving 5 mg of standard substance in 100 μL 0.01 mol L^{-1} HCl solution before diluting to 5 mL by deionized water. All solutions were stored at −20 °C until use.

The artificial urine reported in the reference [40] was used in recovery tests. The artificial urine consisted of 19.4 mg mL^{-1} of urea, 8.0 mg mL^{-1} of NaCl, 1.1 mg mL^{-1} of magnesium sulfate and 0.6 mg mL^{-1} of $CaCl_2$, and the solution was adjusted to pH 8.0 with 1 mol L^{-1} NaOH. Spiked artificial urine solution was prepared containing CAs (NE, EP and DA) in the concentrations of 10, 50 and 200 ng mL^{-1}, and then 2 mL of the spiked solution was mixed with 100 μL of IS (1000 ng mL^{-1}).

2.2. Instruments

A pH meter (Shanghai, China) with a composite electrode was used for the pH values. Special indicator papers indicating pH 6.9–8.4 were bought from SSS Reagent Company (Shanghai, China). An analytical balance (Shanghai, China) was used for weight measurement. A Hitachi S-3400N scanning electron microscope (SEM, Hitachi, Tokyo, Japan) was used at an acceleration voltage of 20.0 kV. Magnetic measurement was carried out using a 7407 vibrating sample magnetometer (Lakeshore, Columbus, OH, USA) at room temperature.

2.3. Chromatography Conditions

A Thermo Scientific UltiMate 3000 ECD UHPLC system based on a glass carbon electrode detector (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Kromasil C_{18} column (5 μm, 250 mm × 4.6 mm) was used. The mobile phase was isocratic elution and consisted of acetonitrile and 0.7% NaH_2PO_4 in water (5.5:94.5, *v*/*v*) with addition of citric acid (35 mmol L^{-1}), EDTA (0.25 mmol L^{-1}) and 1-heptane sulfonic acid sodium salt (2 mmol L^{-1}). The pH of the mobile phase was adjusted to pH 4.0 by adding saturated NaOH solution. The run was performed at 1.0 mL min^{-1} with the column oven temperature at 30 °C. The injection volume was 20 μL and the working potential was 700 mV.

2.4. Preparation of Phenylboronic Acid-Functionalized Fe_3O_4-NH_2@Au (Fe_3O_4-NH_2@Au-MPBA)

The Fe_3O_4 NPs were prepared through reducing the solution of Fe (II) and Fe (III) chlorides of the molar ratio 1:2 with 30% ammonia solution. Appropriate Fe_3O_4 NPs were modified by APTES through stirring in 10% (*v*/*v*) APTES solution in absolute ethyl alcohol for 12 h. The obtained Fe_3O_4 NPs modified by -NH_2 groups (Fe_3O_4-NH_2) were magnetically separated, washed with ethanol and dispersed in ethanol.

Next, 500 mg Fe_3O_4-NH_2 NPs, 50 mL of 2% $HAuCl_4$ and 300 mL distilled water were mixed for over 10 min by sonication, and then 50 mmol L^{-1} KBH_4 solution was dropped into the above mixture under ultrasound until the color changed to purple. Another 5 min of sonication was performed. The Fe_3O_4-NH_2@Au NPs were obtained by magnetic separation.

An amount of 500 mg Fe_3O_4-NH_2@Au NPs was added to 100 mL 1 mg mL^{-1} of MPBA solution under stirring to complete the covalent binding between the Au shell and sulfydryl (-SH). Fe_3O_4-NH_2@Au-MPBA composites were obtained, then separated and washed with distilled water based on external magnetic field. After that, the magnetic nanocomposites were dried in a vacuum oven at 40 °C for 24 h.

2.5. Sample Collection

To investigate the suitability of the proposed method, a pilot study was conducted by analyzing urinary samples from healthy volunteers. The urine samples from 6 healthy children aged 4–6 years (3 boys and 3 girls). Their morning urine samples were collected into sterile urine cups. They were then immediately transferred to polypropylene tubes and stored in a –20 °C refrigerator.

The study was carried out according to the principles of the Declaration of Helsinki (World Medical Association 2008). Written informed consent was obtained from the legal guardians of the volunteers. This study was approved by the Ethics Committee of Zhongda Hospital Affiliated to Southeast University.

2.6. Sample Pretreatment

When analyzing samples, the stored 2.5 mL urine samples were thawed by incubation at 37 °C for 5 min and centrifuged for 5 min at 10,000 rpm. Then, 2 mL of supernatant, 100 μL of 1 μg mL^{-1} DHBA and 5 mg Fe_3O_4-NH_2@Au-MPBA NPs were added into a glass spawn bottle, and the pH was adjusted to 8.0 by adding moderate amounts of 0.01 mol L^{-1} NaOH. After that, the mixture was stirred for about 10 min at room temperature, and the supernatant was removed by external magnetic field. An amount of 200 μL of 0.5% H_3PO_4 solution (pH = 2.0) was added for desorbing the analytes. 20 μL of eluent was injected into HPLC for analysis. The process schematic for the preparation of the magnetic Fe_3O_4-NH_2@Au-MPBA NPs and the pretreatment of samples are shown in Figure 2.

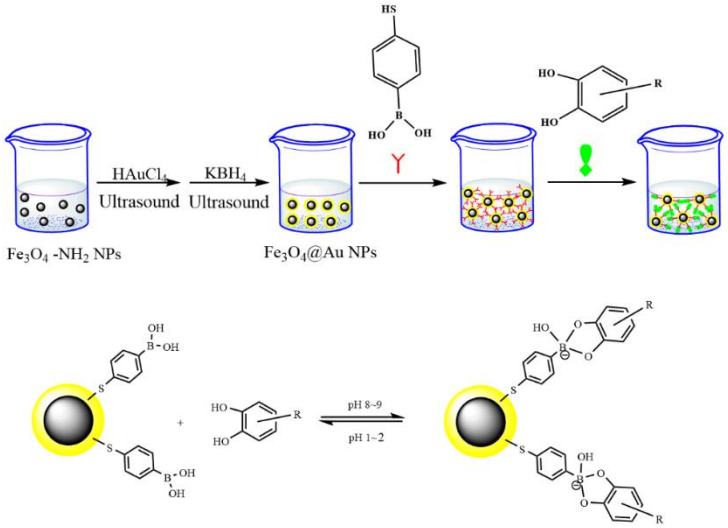

Figure 2. Schematic representation for the preparation of Fe_3O_4-NH_2@Au-MPBA NPs and the extraction of CAs.

3. Results and Discussion

3.1. Characterization

The morphologies of Fe_3O_4-NH_2@Au NPs were examined by SEM at 20.0 kV (8.2 mm, 40.0k SE) (Figure 3A). The size distribution of Fe_3O_4-NH_2@Au NPs is shown in Figure 3B, where we can see that the size of Fe_3O_4-NH_2@Au NPs was uniform, and the average particle size was about 150 nm. The energy-dispersive spectroscopy (EDS) results showed the existence of Fe, C, Si and O elements in Fe_3O_4-NH_2 samples (Figure 3C). Moreover, the Au elements could be found in Fe_3O_4-NH_2@Au material (Figure 3D), which indicated that Fe_3O_4-NH_2@Au NPs were successfully prepared.

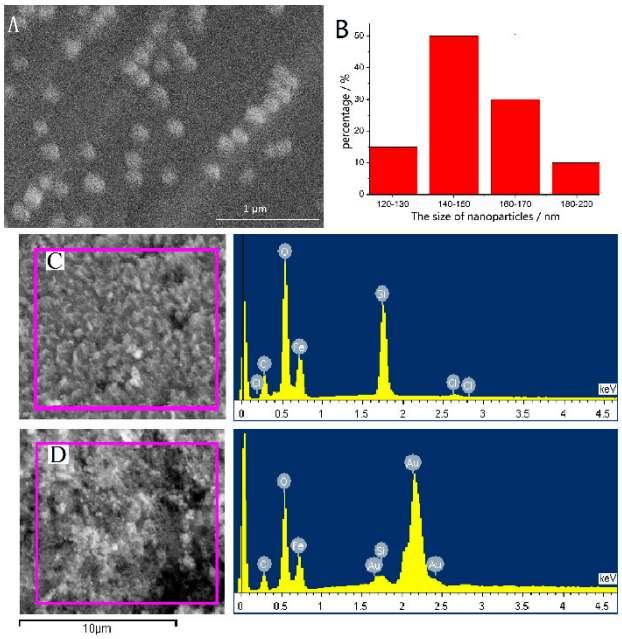

Figure 3. SEM (**A**) and the size distribution (**B**) of Fe_3O_4-NH_2@Au NPs. The EDSs of Fe_3O_4-NH_2 (**C**) and Fe_3O_4-NH_2@Au NPs powder (**D**).

The profile of magnetization loops in Figure 4 revealed that the Fe_3O_4-NH_2 and Fe_3O_4-NH_2@Au NPs are superparamagnetic [29]. The magnetization saturation (Ms) values of Fe_3O_4-NH_2 and Fe_3O_4-NH_2@Au NPs were determined as 32.1 and 22.0 emu/g, respectively. The Fe_3O_4-NH_2@Au NPs showed slightly lower Ms values than that of Fe_3O_4-NH_2 due to the surface conjugation of Au NPs on Fe_3O_4-NH_2.

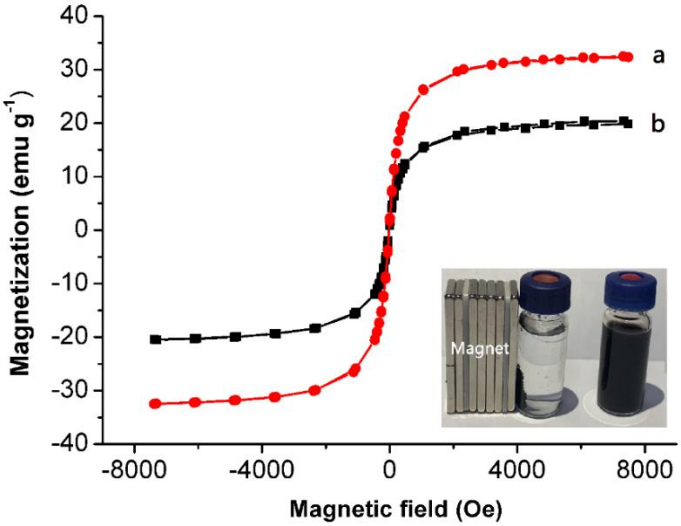

Figure 4. VSMs of Fe_3O_4-NH_2 (**a**) and Fe_3O_4-NH_2@Au NPs (**b**). The two bottles in the inset are both Fe_3O_4-NH_2@Au NPs samples.

In the inset, the two bottles are both Fe_3O_4-NH_2@Au NPs suspension solution. The composite NPs were able to be magnetically separated by an external magnetic field, which indicated that the enrichment and separation of Fe_3O_4-NH_2@Au NPs during the extraction process can be regulated by an external magnetic field.

3.2. Optimization of the Extraction Condition

3.2.1. Optimizing the Mass Ratio of Au^{3+} and Fe_3O_4-NH_2

The effect of the mass ratios of Fe_3O_4-NH_2 and Au^{3+} on magnetic separation time was studied. As shown in Figure 5A, when the mass ratio of Fe_3O_4-NH_2 and Au^{3+} was 3:1, the magnetic separation time was less than 2 minutes; however, too little Au^{3+} may reduce the functionalized molecule numbers of MPBA, which would reduce the sites of interaction for targets. With the increase in Au^{3+}, the magnetic separation time was gradually prolonged. However, when the mass ratio was 1:2, the gold nanoparticles were not completely wrapped on the surface of Fe_3O_4-NH_2 NPs, which was very wasteful for Au^{3+}. When the mass ratio was 1:1.5, the magnetic separation time was 15 minutes, which was too long for rapid pretreatment for samples. Therefore, the mass ratio of 1:1 between Fe_3O_4-NH_2 NPs and Au^{3+} was chosen.

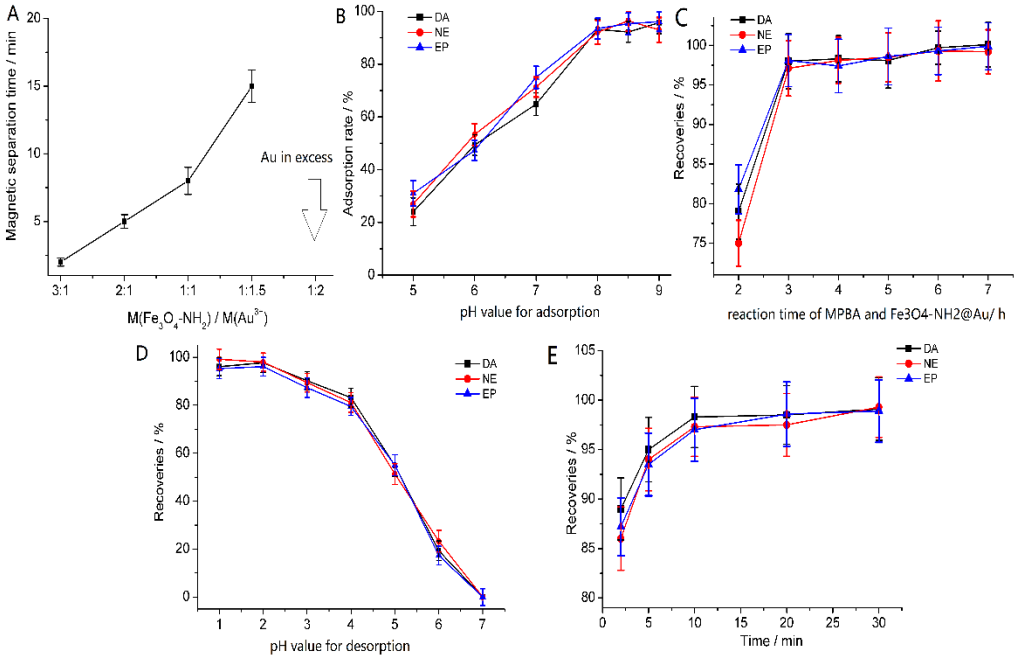

Figure 5. Optimizing the mass ratio of Fe_3O_4-NH_2 and Au^{3+} (**A**), the pH value of adsorption solution (**B**), the reaction time of MPBA and Fe_3O_4-NH_2@Au NPs (**C**), the pH value of desorption solvent (**D**) and elution time (**E**).

3.2.2. Optimizing the pH Value of the Adsorption Solution

The pH value of adsorption solution was a critical factor for boronate affinity. Most boronic acids are generally weak acids, having a pKa values of 8.0–9.0. With pH less than the pKa value, most of them still exist in trigonal form [-$B(OH)_2$], which cannot react with *cis*-diol groups [41]. For phenylboronic acids, when the pH is greater than the pKa value of the phenylboronic acid ligand, the phenylboronic acid group is transformed to tetrahedral anionic form [-$B(OH)_4^-$] under alkaline conditions [35]. Subsequently, [-$B(OH)_4^-$] bonds with cis-1,2-diol units to form a stable 5-membered cyclic ester. This is in good agreement with the results obtained from MPBA functionalized Fe_3O_4-NH_2@Au. In this work, we studied the effect of adsorption pH value (5.0–9.0) on adsorption performance, and the results are shown in Figure 5B. As described above, the phenylboronic acids were the sole functional group on Fe_3O_4-NH_2@Au nanoparticles, and thus the adsorption rates of adsorbents for the three catecholamines were pH-dependent. The adsorption rates improved when the pH value increased from 5.0 to 8.0 and then remained almost unchanged. Thus, pH 8.0 was selected for adsorption.

3.2.3. Optimizing Reaction Time of MPBA and Fe_3O_4-NH_2@Au

The interaction time between MPBA and Fe_3O_4-NH_2@Au NPs is very important, as it could impact the adsorption performance of the magnetic NPs for catecholamines. The relationships between reaction time (2–7 h) and extraction efficiency were explored. As shown in Figure 5C, when reaction time was more than 3h, the extraction efficiency remained nearly unchanged. Thus, 3 h was chosen as the reaction time of MPBA and Fe_3O_4-NH_2@Au NPs.

3.2.4. The pH Value of Desorption Solvent

The pH value of the eluent has an important effect on the formation of boronate ester, because the reversible covalent interactions could be adjusted by pH between the positions of the cis-diol moieties of the targets and the hydroxyl groups of the boronic acid ligand. Acidic environments can break the ester bond and make the target dissociate from Fe_3O_4-NH_2@Au-MPBA NPs. The pH value (1.0–7.0) was investigated for the eluent. With the decrease in pH value of the eluent, the extraction recovery of the targets increased gradually. When the pH value was 2.0, the extraction recovery of the CAs reached the maximum. As the pH value continued to decrease, the recoveries almost remained unchanged. Therefore, pH 2.0 was chosen as the acidity value for the eluent (Figure 5D).

3.2.5. Exploring the Elution Time

In order to obtain the best extraction performance, different elution time from 2 to 30 min was optimized. Figure 5E demonstrated the relationship between the elution time and the recoveries. When elution time was shorter than 10 min, the extraction recovery increased with the extraction time increasing. When elution time was longer than 10 min, the recovery of CAs was almost no longer changed. Therefore, 10 min was chosen as the final elution time.

3.3. Method Evaluation

To test the linearity, we analyzed the mixed solutions of NE, EP and DA at the concentrations of 2.5, 5.0, 10.0, 20.0, 100.0 and 500.0 ng/mL for each target, which were prepared using artificial urine. Quantification was worked out using internal standard (IS) method. The calibration equation was calculated by the HPLC-ECD peak areas ratio and the concentration ratios of analytes and IS. The analysis performances of the established method for the detection of catecholamines are summarized in Table 1.

Table 1. Parameters for methodological validation for catecholamines in artificial urine.

Catecholamines	Linear Range (ng mL^{-1})	r^2	Spiked Concentration (ng mL^{-1})	Recovery (%)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Intraday (RSD, %)	Interday (RSD, %)
DA	2.5–500.0	0.9907	10.0	85.4	0.60	2.0	8.2	9.3
			50.0	93.2			7.7	8.8
			200.0	95.7			6.1	7.4
NE	2.5–500.0	0.9911	10.0	88.1	0.39	1.3	7.9	11.5
			50.0	96.3			8.4	8.1
			200.0	97.1			6.6	9.0
EP	2.5–500.0	0.9935	10.0	89.6	0.27	0.9	10.7	8.6
			50.0	98.3			8.3	7.9
			200.0	105.2			8.9	8.1

The intra-day and inter-day precision and accuracy were evaluated for artificial urine samples spiked with catecholamines at the concentrations of 10, 50 and 200 ng mL^{-1} based on the above pretreatment method. The limits of detection (LOD), defined as the signal-to-noise ratio of 3:1, and limits of quantification (LOQ), defined as a signal-to-noise ratio of 10:1, were calculated; the results are listed in Table 1. Results showed that good linearity of the targets was achieved in the range of 2.5–500.0 ng mL^{-1} with the correlation coefficients (r^2) of 0.9911, 0.9935 and 0.9907 for NE, EP and DA, respectively. The LOQ were 1.3, 0.9 and 2.0 ng mL^{-1} (S/N = 10), and LOD (S/N = 3) were 0.39, 0.27 and 0.60 ng mL^{-1} for NE,

EP and DA, respectively. The RSDs (relative standard deviations) for the catecholamines were from 6.1% to 10.7% for intra-day determination (n = 5) and from 7.4% to 11.5% for inter-day determination (n = 5).

The comparison of recoveries obtained by the present method and a reference method [42] has been added in Table S1 in the Supplementary. The results showed that the prepared method has good extraction recoveries for catecholamines.

3.4. Reproducibility and Stability

The relative standard deviation (RSD, %) of adsorption quantities was 8.1% for six batches of Fe_3O_4-NH_2@Au-MPBA NPs, and 7.3% for one batch used 10 times. The adsorption capacities of Fe_3O_4-NH_2@Au-MPBA NPs for catecholamines decreased by approximately 7.5% after one month. The data above showed that the prepared magnetic composite nanoparticles were a reliable and stable adsorbent for catecholamines.

3.5. Application of the Method to Real Samples

To evaluate the application of the prepared magnetic Fe_3O_4-NH_2@Au-MPBA NPs, urine samples were analyzed using internal standard method. As shown in Figure 6, A was the liquid chromatogram of CAs at 20 ng mL^{-1} in artificial urine with the extraction by magnetic NPs. The liquid chromatogram of a real urine sample (Figure 6B) without the pretreatment by Fe_3O_4-NH_2@Au-MPBA NPs exhibited many impurity peaks. Figure 6C was the liquid chromatogram of a real urine sample with the pretreatment by Fe_3O_4-NH_2@Au-MPBA NPs, where we can find fewer impurity peaks compared with Figure 6B. The results showed that Fe_3O_4-NH_2@Au-MPBA NPs have a highly selective adsorption for catecholamines.

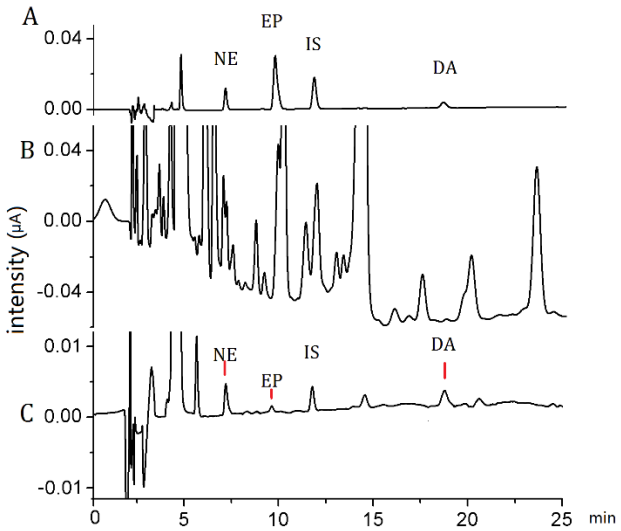

Figure 6. Liquid chromatograms of the mixed standard solution of CAs in artificial urine at 20 ng mL^{-1} with the extraction by magnetic NPs (**A**), (**B**) urine sample without extraction by magnetic NPs, (**C**) real urine sample with extraction by magnetic NPs.

First morning urinations, free of interference of medications and foods, were collected. Free catecholamines were measured in the morning urine samples from six healthy children using our developed method. The concentrations of CAs (NE, EP and DA) of the samples detected by the prepared method are shown in Table 2. The results showed that Fe_3O_4-NH_2@Au-MPBA NPs coupled with HPLC-ECD could be successfully used for the detection of CAs.

Table 2. The concentration of CAs (NE, EP, DA) in real urine samples (M ± SD, *n* = 3).

Samples	Level of Catecholamines (M + SD, ng/mL) (*n* = 3)		
	NE	EP	DA
1	13.2 ± 0.7	10.8 ± 0.3	81.1 ± 9.9
2	26.3 ± 1.1	25.3 ± 1.5	99.1 ± 10.1
3	65.8 ± 5.4	21.1 ± 1.7	279.3 ± 21.6
4	52.6 ± 4.1	43.2 ± 3.5	135.1 ± 14.7
5	65.8 ± 6.7	22.6 ± 1.9	9.0 ± 0.8
6	11.2 ± 0.8	2.7 ± 0.1	15.1 ± 1.3

4. Conclusions

Fe_3O_4-NH_2@Au-MPBA magnetic nanocomposites were synthesized in a simple method first and employed as adsorbents for the selective extraction of NE, EP and DA based on external magnetic field. 4-MPBA was modified on the surface of Fe_3O_4-NH_2@Au NPs through Au–S bond. The interaction between catecholamines and Fe_3O_4-NH_2@Au-MPBA magnetic NPs is based on the covalent bond of borate ester. The adsorption and desorption of catecholamines could be easily achieved through flexibly adjusting the pH value of solution. The SPE based on magnetic nanocomposite coupled with HPLC-electrochemical detection for the separation and analysis of catecholamines in human urine exhibited good sensitivity and selectivity.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/separations8110196/s1, Figure S1: The SEM graph of PS-PCE, Table S1: The comparison of the extraction recoveries for the mixed catecholamines standard in artificial urine at the concentration of 100 ng/mL extracted by the present method and a reference method.

Author Contributions: Methodology, Q.H., Y.C., Y.Z. and H.Z. (Hua Zhang); formal analysis, Q.H.; resources, Y.Z., H.Z. (Huaiyuan Zhu). and X.K.; data curation, Q.H. and X.W.; writing—original draft preparation, Q.H.; writing—review and editing, X.K. funding acquisition, X.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Zhongda Hospital Affiliated to Southeast University (protocol code 2019ZDKYSB083 and 8 February 2019).

Informed Consent Statement: Informed consent was obtained from all subjects and guardians involved in the study. Written informed consent has been obtained from the patient(s) and the guardians to publish this paper.

Data Availability Statement: The data presented in this study is available in supplementary material.

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Conflicts of Interest: All authors have read this paper and approved to submit it to this journal. There are no conflicts of interest for any authors in relation to the submission.

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