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A Convenient Method for Catecholamines and Their Metabolites Extraction and Analysis with HPLC-ECD in Infants' Urine

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Dear Editor,

We would like to submit the enclosed manuscript entitled "A Convenient Method for Catecholamines Neurotransmitters and Their Metabolites Extraction and Analysis with High-pressure Liquid Chromatography in Infants'Urine", which we wish to be considered for publication in JoVE.

The work described has not been submitted elsewhere for publication, in whole or in part, and all the authors listed have approved the manuscript that is enclosed. We believe that two aspects of this manuscript will make it interesting to general readers of your journal.

First, we developed a simple and convenient solid-phase extraction coupled to HPLC-ECD for determination of three monoamine neurotransmitters and two of their metabolites in infants' urine simultaneously. The column packed with electrospun nanofibers used to purify the urine sample is highly effective and selective.

Second, the established method was applied into assessing the differences of urinary catecholamines between the high-risk infants with perinatal brain damage and healthy controls. Comparative analysis reveals a significant difference in urinary MHPG between the two groups, indicating that MHPG may be a candidate marker for early diagnosis of cases at risk for brain damage in infants. Indicating that the method in the study is suitable for clinical use, and promising in exploring the early diagnosis of neurosystem diseases of high-risk infants via urine medium.

Thank you very much for your time and consideration.

Sincerely yours,

Li Xie

TITLE:

A Convenient Method for Extraction and Analysis with High-Pressure Liquid Chromatography of Catecholamine Neurotransmitters and their Metabolites

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KEYWORDS:

Catecholamines, polymeric crown, electrospun nanofiber, packed-fiber solid-phase extraction (PFSPE), high-pressure liquid chromatography with electrochemical detection (HPLC-ECD), high-risk infants

SHORT ABSTRACT:

We present a convenient solid-phase extraction coupled to high-pressure liquid chromatography (HPLC) with electrochemical detection (ECD) for simultaneous determination of three monoamine neurotransmitters and two of their metabolites in infants' urine. We also identify the metabolite MHPG as a potential biomarker for the early diagnosis of brain damage for infants.

LONG ABSTRACT:

The extraction and analysis of catecholamine neurotransmitters in biological fluids is of great importance in assessing nervous system function and related diseases, but their precise measurement is still a challenge. Many protocols have been described for neurotransmitter

measurement by a variety of instruments, including high-pressure liquid chromatography (HPLC). However, there are shortcomings, such as complicated operation or hard-to-detect multiple targets, which cannot be avoided, and presently, the dominant analysis technique is still HPLC coupled with sensitive electrochemical or fluorimetric detection, due to its high sensitivity and good selectivity. Here, a detailed protocol is described for the pretreatment and detection of catecholamines with high pressure liquid chromatography with electrochemical detection (HPLC-ECD) in real urine samples of infants, using electrospun composite nanofibers composed of polymeric crown ether with polystyrene as adsorbent, also known as the packed-fiber solid phase extraction (PFSPE) method. We show how urine samples can be easily precleaned by a nanofiber-packed solid phase column, and how the analytes in the sample can be rapidly enriched, desorbed, and detected on an ECD system. PFSPE greatly simplifies the pretreatment procedures for biological samples, allowing for decreased time, expense, and reduction of the loss of targets.

Overall, this work illustrates a simple and convenient protocol for solid-phase extraction coupled to an HPLC-ECD system for simultaneous determination of three monoamine neurotransmitters (norepinephrine (NE), epinephrine (E), dopamine (DA)) and two of their metabolites (3-methoxy-4-hydroxyphenylglycol (MHPG) and 3,4-dihydroxy-phenylacetic acid (DOPAC)) in infants' urine. The established protocol was applied to assess the differences of urinary catecholamines and their metabolites between high-risk infants with perinatal brain damage and healthy controls. Comparative analysis revealed a significant difference in urinary MHPG between the two groups, indicating that the catecholamine metabolites may be an important candidate marker for early diagnosis of cases at risk for brain damage in infants.

INTRODUCTION:

Catecholamine neurotransmitters and their metabolite contents in body fluids can affect neural function and affect the balance of response-to-stimulus states to a large extent¹. Abnormalities may cause a variety of diseases, such as pheochromocytoma, ganglioneuroma, neuroblastoma, and neurological disorders^{1,2}. The extraction and determination of catecholamines in body fluids is meaningful to the diagnosis of the relevant diseases. However, catecholamines in biological samples exist in low concentrations and are easily oxidized. Furthermore, they are very difficult to elute because of the large amount of interference in the medium³. Thus, simultaneous detection of catecholamines in biological fluids is still a challenge.

There have been reviews showing that urinary catecholamines can be a measure of stress, and that their levels are important biological markers responding to tactile stimulation processing in newborns⁵. According to the research, all infants who have suffered from premature incidents are at risk for brain injury⁴⁻⁶, and injury may cause abnormal release of catecholamines and related matters into the fluids. There do exist advanced magnetic resonance techniques that can

79 detect brain damage in earlier phases^{7,8}. However, within the first 48 h, an abnormal
80 neurodevelopmental process will cause permanent brain injury that won't be evident in medical
81 images¹¹. Besides, the high cost and scarce instrument resources, along with other factors, makes
82 it impossible for all neonatal units to have access to these specialized neuro-imaging techniques.
83 However, the use of an easily approachable and practical biomarker (such as catecholamines and
84 their metabolites) could overcome these shortcomings, and the screening of a biomarker in
85 human fluids may help in the early diagnosis of brain injury and lead to prompt identification of
86 new-born infants needing neuroprotection⁹. The catecholamines in urine can be an easy and
87 obvious index, because of the direct correlation between the amount of them released into fluids
88 and neuroactivity function.

90 Among biological fluids, cerebrospinal fluid (CSF) and plasma samples are not easy to get via
91 existing traumatic procedures, and it is also very difficult to get rid of interference due to
92 adhesive protein and other impurities, leading to a troublesome and time-consuming sampling
93 process that is unsuited for repeated detection. Also, for children, it is almost impossible to get
94 the samples in a traumatic fashion. Therefore, urinary sampling is better than the other forms
95 of sampling, as it is non-invasive, easy to operate, and can be repeatedly done. Urine samples
96 are abundant and easy to store, and show great advantages over the other forms of biological
97 samples.

99 The main methods to quantify catecholamines in biological fluids include radioenzymic assays¹⁰,
100 enzyme-linked immune-sorbent assays¹¹, voltammetry¹² and thermal lens spectrometry¹³. But
101 shortcomings exist, such as complicated operations and hard-to-detect multiple targets. Today,
102 the dominant analysis technique is high-performance liquid chromatography (HPLC)¹⁴, coupled
103 with sensitive electrochemical¹⁵ or fluorimetric detection¹⁶, because of its high sensitivity and
104 good selectivity. With tandem mass spectrometry technology, such as liquid chromatography
105 /mass spectrometry (LC/MS) and liquid chromatography/mass spectrometry/mass
106 spectrometry (LC/MS/MS), the analysis and quantification of the neurotransmitters can achieve
107 high accuracy and specificity^{17,18}. However, the MS technique requires expensive
108 instrumentation as well as substantially qualified manpower, making the method difficult to
109 apply universally in most conventional laboratories. HPLC-ECD systems are commonly equipped
110 in most conventional and clinical laboratories, and have thus become a common and good
111 choice for research groups to use for chemical determination, but they require the sample
112 introduced into the system to be clean and of microscale volume¹⁹. Thus, it is of great
113 importance to purify and condense the sample prior to the analysis. The classical method for
114 the purification step is liquid-liquid extraction^{14,15,20} and off-line solid-phase extraction,
115 including activated alumina column^{21,22} and diphenylborate (DPBA) complexation²³⁻²⁶.

Myeongho Lee *et al.* have been using polymer resin chemically modified with crown ether as the adsorbent to selectively extract catecholamines from human urine since 2007²⁷. Also, in 2006, Haibo He *et al.* demonstrated a facile synthesis approach for boronate affinity extraction sorbent by utilizing a functionalizable nanomagnetic polyhedral oligomeric silsesquioxane (POSS) based nanomagnetic composite, and applying it to the enrichment of catecholamines in human urine (noradrenaline, epinephrine and isoprenaline)²⁸. They also took advantage of the nanomaterials to fulfill the work, using a technology called nano-electrospinning and forming the polymer fibrous material in the nanoscale. The electrospinning process can adjust the diameter, morphology, and spatial alignment of the product by controlling the working voltage and changing the content of the spinning solution along with other parameters²⁹. Compared with the conventional SPE cartridge, electrospun nanofibers are highly suitable to extract and enrich target analytes from a complex matrix, as they are equipped with high surface-area-to-volume ratios to adsorb the analytes with high efficiency, and exhibit more easily-controlled surface chemical properties, allowing handy attachment of the target compounds. These properties make them good choices for SPE adsorbents, greatly reducing the solid phase and desorption solvent amount³⁰⁻³³. For catecholamines in urine samples, electrospun nanofibers composed of apolymeric crown ether with polystyrene (PCE-PS) were used to selectively extract three catecholamines (NE, E, and DA)³⁴. The paper indicated that the selective crown ether adsorbed the targets of NE, E, and DA, which was based on its correct geometry for binding catecholamines via forming hydrogen bonds. The results displayed the material crown ether effectively, removing other interfering compounds contained in biological samples. Inspired by this report, a novel method was developed for the selective extraction of the catecholamines by use of electrospun composite nanofibers composed of PCE-PS.

In this paper, the method reported previously³⁴ was improved and employed not only to successfully analyze E, NE, and DA, but also their metabolites, MHPG and DOPAC, in urine. We also explore new possibilities for the mechanism of the adsorption process. The method shows satisfying extraction efficiency and selectivity for the five analytes, and the method was verified in the analysis of urine from high-risk infants with perinatal brain damage and healthy controls.

PROTOCOL:

Informed consent from the parents was obtained, and institutional review board approval was obtained for the study. The study was performed in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Caregivers of all participants provided written consent for being enrolled in the study. Ethical committee approval from Zhongda Hospital, affiliate with Southeast University, was also obtained.

1. Preparation of the Columns and Solutions Needed for the Extraction and Determination of Catecholamines

1.1. Prepare the PFSPE column. Divide 1-2 mg of PCE-PS nanofibers into aliquots, and use a fine steel rod with 0.5 mm diameter to compress them orderly into the end of a pipette tip with a volume of 200 μ L.

1.2. Prepare the artificial urine by weighing 2.427 g urea, 0.034 g uric acid, 0.09 g creatinine, 0.297 g trisodium citrate, 0.634 g sodium chloride, 0.45 g potassium chloride, 0.161 g ammonium chloride, 0.089 g calcium chloride dihydrate, 0.1 g magnesium sulfate heptahydrate, 0.034 g sodium bicarbonate, 0.003 g sodium oxalate, 0.258 g sodium sulfate, 0.1 g sodium dihydrogen phosphate, and 0.011 g disodium hydrogen phosphate, and dissolve the above chemicals into 200 mL deionized water.

1.3. Prepare 2 mg/mL stock solution of diphenylborate (DPBA) solution by dissolving 2 mg of the compound into 1 mL of distilled water. Store the solution in the dark at 4 $^{\circ}$ C.

1.4. Prepare 1 mg/mL of internal standard (IS) solution. Dissolve 1 mg of 3,4-dihydroxybenzylamine hydrobromide (DHBA) into 1 mL of distilled water to get stock solution. Store the standard solution in the dark at 4 $^{\circ}$ C. Dilute the stock solution in water to 100 ng/mL before use.

1.5. Analyte standard

NOTE: The chemical structure and properties of catecholamines are instable, and they easily decompose. The preparation process of standards has to be very fast and must prevent exposure to direct sunlight.

1.5.1. Measure 1.0 mg of NE, E, DA, MHPG, DOPAC and DHBA in separate 1.5 mL microcentrifuge tubes. For NE, DA, MHPG, DOPAC and DHBA, add 1.0 mL distilled water to dissolve. For E, add 0.01 M hydrochloric acid solution to dissolve.

1.5.2. Oscillate the prepared standards in the dark at a high speed until analytes dissolve completely. This is the primary stock; store at -20 $^{\circ}$ C for up to several weeks.

1.5.3. Prepare the secondary 1,000 ng/mL analyte stocks. For NE, E, DA, DOPAC and MHPG, transfer 5 μ L of each primary analyte stock into 4,975 μ L of distilled water in a 5 mL centrifuge tube, and store it in the dark at 4 $^{\circ}$ C until use. Prepare these solutions fresh daily. For DHBA,

transfer 5 μ L of primary stock into 4,995 μ L of distilled water in a 5 mL centrifuge tube, and store it in the dark separately at 4 °C.

1.5.4. Make further dilutions with the secondary analyte stock to create a standard curve (e.g., **Supplementary Table 2**). Store solutions in the dark at 4 °C and prepare fresh daily.

1.5.5. Test the optimal voltage of ECD detector using the standard stock with proper concentration. Vary the voltage to find a value where the analytes have the best peak appearance.

1.6. Prepare eluant containing 30% phosphoric acid, 15% acetonitrile, and 55% distilled water. For 10 mL of eluant solvent, use 5.5 mL of distilled water, and add 1.5 mL of acetonitrile and 3 mL of phosphoric acid drop by drop into the water.

2. Preparation of Real Urine Samples and Mobile Phase

2.1. Have mothers collect the first morning urine of their infants using aseptic urine cups. Transfer the samples into polypropylene tubes and label immediately. Then, store the samples in a -20 °C freezer.

2.2. Vortex and centrifuge the urinary samples at 1,510 x g for 10 min at room temperature (RT) to get rid of most particulate interference. Discard the sediment and gather the supernatants for further experiments. In order to extract analytes effectively, proceed to PFSPE pretreatment (step 3) immediately after centrifuging.

2.3. Prepare the mobile phase

2.3.1. Prepare a clean bottle, at least 1 L. The composition of mobile phase is listed in **Supplementary Table 1**; for 1 L mobile phase, measure 6.7242 g of citric acid, 93.06 mg of ethylene diamine tetra acetic acid (EDTA) disodium salt, 7.02 g of monometallic sodium orthophosphate, 404.5 mg of 1-heptanesulfonic acid sodium salt, and 3.5 g of sodium hydrate into the bottle. Add 40 mL acetonitrile and distilled water to 1,000 mL. Agitate and vibrate ultrasonically for 15 min until the matter in the solution is all dissolved.

2.3.2. Using a pH meter with a glass electrode, adjust the pH value of the mobile phase to 4.21 with a saturated sodium hydroxide solution.

2.3.3. Filter the mobile phase with a 0.45 μ m polyvinylidene fluoride microporous membrane and a vacuum suction device to get rid of impurities.

233
234 2.3.4. Use ultrasonic vibration for 15 min to degas the mobile phase each time before use.
235

236 **3. PFSPE Extraction and HPLC Analysis**

237
238 3.1. Activate the nanofibers. Press 100 μ L of methanol and 100 μ L of water sequentially through
239 the PFSPE column using a 5 mL syringe in a slow, dropwise manner.
240

241 3.2. Mix 100 μ L urine sample with 100 μ L 2 mg/mL DPBA solution and 30 μ L 100 ng/mL of DHBA
242 solution (IS, internal standard) in a 0.5 mL EP tube, then transfer the mixed solution to the
243 PFSPE column. Press the mixed sample solution through the PFSPE column with a 5 mL gastight
244 plastic syringe using the force of the air pressure.
245

246 3.3. Leach the column three times by loading 100 μ L of DPBA solution (2 mg/mL) into the SPE
247 column, and push the solution slowly through the cartridge with air pressure using a 5 mL
248 gastight plastic syringe.
249

250 3.4. Load 50 μ L of the eluant onto the PFSPE column, and push it through the column, collecting
251 the eluate with a 0.5 mL EP tube.
252

253 3.5. Turn on the HPLC degasser to degas the air in the system. Prior to sample analyses, the
254 system should run for more than 0.5 h with the mobile phase to equilibrate and reduce
255 baseline noise. See **Supplementary Table 1** showing the setup parameters of the HPLC system.
256

257 3.6. Sample 20 μ L of the eluate using an automatic sampler, and then inject it into the HPLC-
258 ECD system.
259

260 3.7. When the runs are complete, turn off the detector cell using the detector interface. DO
261 NOT turn off the cell with the switch at the back of the detector, as this could damage the
262 instrument.
263

264 3.8. Manually change the mobile phase composition to 10% methanol and 90% water. Run for
265 at least 30 min. Then, manually change the mobile phase to HPLC-grade methanol. Run for
266 about 15 min to protect the system in methanol. Failure to run this step following the
267 recommended running time could result in damage to the column and the detector. Turn off
268 the flow, then turn off the degasser.
269

270 **4. Phenylboronic Acid Cartridges (PBA) Extraction**

271

PBA cartridge extraction procedures were similar to the scheme in Kumar *et al.* (2011)²⁵. All solutions are pushed through the PBA cartridge (100 mg, 1 mL) with air forced by a syringe.

4.1. Condition the cartridge with 1 mL 80:20 acetonitrile-water (v/v) containing 1% formic acid and 1 mL of 50 mM phosphate buffer (pH 10) sequentially.

4.2. Press the buffered urine sample (1 mL urine and 2 mL phosphate buffer, pH 8.5) through the PBA cartridge.

4.3. Wash the cartridge with 1 mL 50:50 v/v acetonitrile-phosphate buffer (10 mM, pH 8.5).

4.4. Elute the cartridge with 1 mL acetonitrile-water (80:20 v/v) containing 1% formic acid.

5. Identification and Quantification of Catecholamines

5.1. Linearity

5.1.1. Dilute the secondary analytes stock with artificial urine to six concentrations (1.5, 3, 12, 25, 50, and 100 ng/mL); the dilution volume of artificial urine follows **Supplementary Table 2**. Make three parallel samples with each concentration to get 18 analyte experimental solutions for constructing calibration curves.

5.1.2. Dilute DHBA secondary stock with artificial urine 10-fold to get 100 ng/mL experimental solution.

5.1.3. Pretreat all the analyte solutions from 5.1.1, according to step 3 (PFSPE extraction procedures). As in Step 3, inject 20 µL of each corresponding eluate into the HPLC-ECD system to get an HPLC chromatogram.

5.1.4. Construct calibration curves of the five analytes by plotting the ratio of peak area (targets/IS) as the Y axis against the ratio of concentrations (targets/IS) as the X axis, as shown in **Supplementary Figure 1**.

5.2. LOD and LOQ value for sensitivity

5.2.1. Inject 20 µL of the blank artificial urine into the HPLC-ECD system (as in Step 3), to obtain the HPLC chromatogram of the sample.

5.2.2. In the chromatogram from 5.2.1, collect 11 blank signal values, and calculate the mean value X_b and standard deviation S_b . Calculate the minimum signal of a substance that can be detected at a certain level of confidence, X_L , as $X_L = X_b + K \cdot S_b$ (K is the coefficient determined by confidence level, S_b reflects noise level of the measuring method and the level of machine noise). Thus, $LOD = (X_L - X_b)/S = (K \cdot S_b)/S$ (S stands for the slope value of the working curve).

5.2.3. Define an S/N of 3:1 ($K=3$) as the limit of detection (LOD), and an S/N of 10:1 ($K=10$) as the limit of quantification (LOQ).

5.3. Evaluate the recoveries

5.3.1. Prepare real and spiked urine samples. Dilute the secondary analytes stock with real urine to three concentrations (5, 50, 100 ng/mL) to obtain the spiked urine samples. Prepare three parallel samples for each analyte solution. Count the spiked concentration as the quantity of target compounds spiked into the urine sample. Define this value as A_s .

5.3.2. Dilute DHBA stock to 100 ng/mL, as in step 5.1.2.

5.3.3. Process each sample solution from 5.3.1 according to step 3 (PFSPE extraction procedures) and inject the 20 μ L of each corresponding eluant into the HPLC-ECD system to get the chromatogram result. The value of analytes will be counted as a quantity of target compounds quantified in the spiked urine sample. Define this value as A_t .

5.3.4. Inject 20 μ L of urine sample into the HPLC-ECD (as in step 3) system to get the chromatogram result. The value of analytes will be counted as an initial quantity of target compounds quantified in the urine sample. Define this value as A_i .

5.3.5. Calculate the quantity of target compounds in the samples from the standard curve equation. The percentage recovery is estimated as methodological recovery % = $(A_t - A_i) \times 100/(A_s)$. Mean values are shown in **Table 1**.

5.4. Evaluate the imprecision

5.4.1. Prepare spiked artificial urine samples to 5, 50, and 100 ng/mL concentrations as in step 5.3.1. Prepare six parallel samples for each analyte solution. Prepare fresh experimental samples every day.

5.4.2. Dilute DHBA stock to 100 ng/mL as in step 5.1.2.

5.4.3. Evaluate the intra-day precision (n=6). Process each sample solution in 5.4.1 according to step 3 and inject the 20 μ L of each correspondent eluant into the HPLC-ECD system to get the chromatogram. Do the same operation six times in the same day.

5.4.4. Calculate the quantity of target compounds in the samples from the standard curve equation. Under the same concentration of the same compound, the relative standard deviation (RSD) of the six assays in one day is determined as intra-day precision. Mean values are shown in **Table 1**.

5.4.5. Evaluate the inter-day precision (n=6). At the same time each day in the six sequential days, prepare spiked artificial urine samples for three concentrations of 5, 50, 100 ng/mL, as in 5.4.1 and 5.4.2., and process each analyte sample solution according to step 3.

5.4.6. Inject the 20 μ L of each corresponding eluate from 5.4.5 into the HPLC-ECD system to get chromatogram results each day. Calculate the quantity of target compounds in the samples from the standard curve equation. Inter-day precision is expressed by the RSD of the assays quantity from the spiked artificial urine samples at the three concentrations in six sequential days. Mean values are shown in **Table 1**.

REPRESENTATIVE RESULTS:

This protocol is a simple and convenient PFSPE method to pretreat urine samples and enrich five catecholamines for detection via an HPLC-ECD system; a diagram of the process is shown in **Figure 1**. The protocol mainly includes four steps—activating, loading, rinsing, and eluting—coupled with a small quantity of PCE-PS nanofibers and a simple solid-phase extraction device. The morphology of PCE-PS nanofibers was assessed using a surface and porosity analyzer (see **Table of Materials**). The textural properties—the BET (Brunauer, Emmett, and Teller) surface area, pore volume and pore size—were 2.8297 m² g⁻¹, 0.009 cm³ g⁻¹, and 12.76 nm, respectively. These data indicate that the material used in the protocol has nanoscale pores on the surface, which may contribute to the high adsorption efficiency and the lowered binding pH in the protocol.

This protocol uses optimized volumes, sample ingredients, leachate, eluant, *etc.*, as well as working pH and time consumed during the procedures. In Chen *et al.*, the eluant was a 12.0 M acetic acid solution because acidic conditions cause the adsorbent to become protonated and positively charged, which is favorable for the elution of CAs^{34,35}. In this study, the eluant recipe was reconditioned to be 30% phosphoric acid, 15% acetonitrile, and 55% distilled water. The final pH value of eluant was adjusted to 3.0. The eluant solvent should be kept in an acidic

environment when eluting, but much more moderate than 12.0 M acetic acid, which may lead to poor peak appearance and damage to the HPLC system.

For identification of the catecholamines, chromatogram retention times of the peaks in the samples with standard solution peaks are compared. **Figure 2** shows examples of HPLC-ECD chromatograms from the various solutions. If the protocol is followed successfully, the chromatographic profiles of three catecholamines and their metabolites should be obtained by HPLC-ECD with clear symmetrical, well defined peaks and with minimal background noise, as illustrated in **Figure 2**. For comparison with the conventional method, a commercial PBA cartridge was selected as a control. In **Figure 2(a–c)**, five target peaks in **(b)** are significantly higher than those in **(c)**, indicating that the PFSPE method is more sensitive than the PBA cartridge method. Also, the DOPAC peak did not show in the PBA cartridge extraction result, indicating that the PBA column was not capable of extracting DOPAC. **Figure 2(d)** depicts the chromatogram of the blank urine sample without any pretreatment, and **Figure 2(e)** shows the chromatograms of the urine sample extracted using PCE-PS composite nanofibers. **Figure 2(f)** shows the chromatograms of the urine sample after the extraction with the PBA column, which also shows no DOPAC extraction consistent with the result in **Figure 2(c)**. The diagram indicates that the PFSPE method could not only extract the targets with good effect, but also could get rid of most of the interference in the urine, giving good peak identification for the target compounds.

Statistical analysis revealed that measurements for the three catecholamines and the two metabolites were reliably reproduced (**Table 1**). All the target compounds showed good linearity between 1.5 and 100 ng/mL ($R^2 > 0.99$), and the standard curve of each analyte can be found in **Supplementary Figure 1**. The curves and R^2 values demonstrate that the analytes have good linearity and relativity within a certain linear range, suitable for the calculation of concentrations of analytes in urine samples. The limits of detection (LOD) ranged from 0.25 to 0.54 ng/mL, and the limits of quantification (LOQ) were 0.83 to 1.81 ng/mL, respectively. The signal-to-noise (S/N) value equalled 3. The range of the methodological recoveries of the five target compounds was from 97.4% (MHPG) to 124.2% (DOPAC), which was satisfactory for the application to the actual samples. The intra-day precision was from 2.7 to 4.8% (expressed as relative standard deviation) and the inter-day precision was 2–8.1%, displaying good precision and repeatability.

For detection of targets in real urine samples, 28 high-risk infants and 22 healthy infants in the division of child care, Suzhou municipal hospital, were recruited. All 50 infants taken into the study were boys. When they were six months old, they were taken to a routine health check in September 2016. All the urine samples were collected and pretreated following the protocol steps 3.1 and 3.2, and the samples were analyzed using the remainder of step 3. The

concentrations were calculated against the calibration curves. Statistical differences were analyzed by analysis of variance (ANOVA). The results can be seen in **Table 2**. The difference of the catecholamines and metabolites between the two groups were compared and analyzed. The *p*-values show that the catecholamines were not significantly different between the high risk and healthy groups, while the metabolite MHPG content was different across these groups ($p = 0.001$). The high-risk infants group had higher amounts of MHPG than the control group (14.8 ± 3.6 ng/mL vs. 1.4 ± 0.2 ng/mL), which means that the level of urinary MHPG may be a potential marker for early identification of high-risk infants.

Figure 3 and **Table 3** describe the classic quantification method for determining monoamine materials, and compare with other methods for which the operation process and figures of interest are given. Compared with classic methods, PFSPE method has advantages like a short timespan (5–10 min), simplified operation process, less organic solvent, and more environmental friendliness with satisfactory methodological parameters. The eluant amount needed is low in volume (50 μ L) and the target enrichment step requires no evaporation, which greatly promotes the detection sensitivity for the target compounds in the urine. Compared to conventional particle-based SPE, this method enhanced the efficiency, simplified the preparation process, and reduced the time of the analysis with acceptable reliability, selectivity, and sensitivity.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic flow chart of PFSPE procedure in the paper and a representation of its device. (1) Gastight syringe, (2) Pipette tip, and (3) Packed nanofibers.

Figure 2: Chromatograms of different samples. (a) Spiked water sample with targets and IS in (100 ng/mL) without extraction. (b) Spiked water sample extracted by the PFSPE method, and (c) by commercial phenylboronic acid (PBA) cartridge. (d) Real blank urine sample without extraction. (e) Real urine sample extracted by the PFSPE method. (f) Real urine sample extracted by commercial PBA cartridge.

Figure 3: Analytical flow chart of determination methods. (a,b) Previously reported classic extraction methods. (a) Extraction by alumina, (b) by DPBA complex, and (c) by the method proposed in this paper.

Supplementary Figure 1: Standard curves and equations. The standard curves for the five analytes are constructed in order to use the equation of the line to calculate the unknown analytes concentration in the sample. (a) NE, (b) E, (c) DA, (d) MHPG, and (e) DOPAC.

Supplementary Figure 2: Boronate affinity interaction between boronic acid and cis-diol group or adjacent two hydroxyl groups. (A) Schematic of the interaction between boronic acids and multiple –OH groups to form five- or six-membered cyclic esters. (B) Cis-diol group or adjacent two hydroxyl groups in red circles depict the major reaction sites for the five analytes to boronic acid compound.

Table 1: Analytical results of the proposed protocol for the determination of three catecholamines and two metabolites with standard solutions.

Table 2: The comparison of concentrations for three catecholamines and two metabolites in urines between healthy infants and high-risk infants. The results were statistically analyzed using analysis of variance (ANOVA) with a significance level of $p = 0.05$ for differences between MHPG content. Means \pm standard deviations are shown.

Table 3: Comparison of this work with studies on pretreatment of monoamines or other related topics in recent years.

Supplementary Table 1: Instrument parameters for detection and quantification of analytes in the paper by HPLC-ECD.

Supplementary Table 2: Preparation of standard curve for five analytes.

DISCUSSION:

The proposed PFSPE method in this paper may be significant and meaningful with respect to its rapidity, simplicity, and convenience. The adsorbents used in the protocol are electrospun nanofibers, which have high surface area-to-volume ratios, and adsorb the analytes with high efficiency. The procedure only needs a few milligrams of nanofiber and a small volume of eluant solvent, and does not require an evaporation step to concentrate the analytes. Here, we have presented a detailed overview of an HPLC-ECD based protocol that will permit new users to establish an effective method for detection and quantification of three catecholamines and two of their metabolites (NE, E, DA, and MHPG, DOPAC).

The superiority of this protocol mainly stems from the four critical steps during the procedures, as shown in **Figure 1**: using PCE-PS nanofibers to capture the target compounds to reduce the size of sorbent to a few milligrams, leading to a rapid adsorption/desorption, and only needing a small volume of eluting fluid (1); adding DPBA into the urine to complex with the analytes to improve their hydrophobicity (2); rinsing the nanofibers with the solution containing DPBA to

retain the analytes and remove the impurities (3); and optimization of the extraction and analysis condition to get good sensitivity and selectivity for the analytes (4).

For step (1), the mechanism of superior performance of adsorbent ability for PCE-PS may attributed to a variety of factors. The crown ether polymers doped in the nanofibers can form a host-guest complex with the guest molecules containing amine groups by H-bonds, such as the catecholamines in this paper. In addition, Hongyou Hu *et al.* and Jishun Chen *et al.* also proposed that boronic acid can bond with nitrogen atoms in the chemical structure through B–N interactions, and that the hydrophobic skeleton can interact with benzene rings and other aliphatic groups of analytes^{36,37}. Also, during the desorption process, the hydrogen bonds and B–N interactions are easily broken at very low pH; thus, eluants with acidic pH are usually suitable for exploration for desorption. Furthermore, there are also several secondary interactions, including hydrophobic, ionic, and hydrogen bonding, which can occur between boronic chemicals and related compounds^{36–38}. All these interactions may contribute to the adsorption of the analytes to the materials.

For step (2), with the help of added DPBA, PCE-PS nanofibers adsorb DPBA-catecholamine complexes rapidly. Zhen Liu *et al.* showed that the boronate affinity materials have emerged as important media for the selective separation and molecular recognition of organic compounds, such as nucleosides, saccharides, glycans, glycoproteins, and so on³⁸. The complexation mainly occurs from the boronate affinity interaction between boronic acid and cis-diol groups, or two adjacent hydroxyl groups, to form five or six membered cyclic esters, as shown in **Supplementary Figure 2A**. **Supplementary Figure 2B** depicts the major reaction sites for the five analytes in this work to the boronic acid compound. When the surroundings become acidic, the boronic acid–cis-diol complex dissociates. The interaction between boronic acid and the cis-diol group or the adjacent two hydroxyl groups exists extensively and relatively strongly.

For step (3), the complex formed between analytes and DPBA can hold them on the adsorbent while other impurities are rinsed from the PCE-PS surface, achieving the best reservation for the targets. For step (4), there has been a report showing that the optimal pH was about 9.0 for the bonding of boric acid and catechol compound³⁹. However, Chen *et al.* explored the best pH value for the complexing of catecholamine-DPBA and found that the adsorption performance of the composite PS-PCE nanofibers for the CAs was optimal when the pH value was changed to be between 6.0 and 7.0^{34,35}. Liu *et al.* implicated that the structure of the supporting materials for boronate acid compounds, especially with spatial confinement of cavities and nanoscale pores, and the B–N ligands formed during the reaction, can also significantly lower the binding pH and enhance binding affinity³⁸. The PCE-PS structure as well as catecholamine structure are all equipped with –NH– groups. Thus, the properties and nanoscale pores of PCE-PS (data as shown in **Representative Results**), as well as B–N ligands formed, may contribute to the

lowered binding pH in the protocol. Thus, the optimal pH value in this protocol can be neutral, which greatly simplifies the procedures and avoids the degradation of the analytes.

The main limitations of catecholamine determination are that their concentrations in biological samples are very low and that their structures are unstable (easily oxidized); furthermore, it is difficult to get rid of the impurities in the media. Thus, for analysis of catecholamine in biological samples, pretreatment, usually by solid-phase extraction, is necessary. The PFSPE method proposed in this paper provided a simple and convenient pre-concentration for the analytes in the urine samples. But, when doing the experiment, the experiment condition must be strictly controlled, by means such as avoiding light exposure and shortening the pre-treatment time as much as possible.

The applicability of the method was evaluated by its use to determine the concentrations of three catecholamines and two metabolites in the urine of healthy infants and high-risk infants. There was a significant difference between the two groups in urinary MHPG. As summarized previously, the MHPG amount in the human body is now mainly reported as an index to reflect noradrenergic neuronal tone, the catecholamine metabolism activity in the central and peripheral nervous systems⁴⁰⁻⁴², and is a useful plasma/urine marker for central NE metabolism⁴³. For high-risk infants, a variety of factors such as hypoxic-ischemic encephalopathy (HIE) causes brain trauma and brain insults, leading to different degrees of childhood neurodevelopmental deficits. This brain damage in turn will lead to the release of neurotransmitters (MHPG included) into body fluids^{44,45}. According to reports by J.W. Maas, there are significant correlations between the brain, CSF, plasma, and urinary concentrations of MHPG^{46,47}. The measurement of total (free + conjugated) MHPG in urine has long been used to assess the metabolism of central NE and peripheral NE metabolism in humans^{41,48,49}. Thus, it may be concluded that high risk infants have noradrenergic neuronal tone damage compared with healthy ones, affecting catecholamine metabolism in the central and peripheral nervous systems. This discrepancy indicates that further research should be done on the relationship between the urinary MHPG level and neurodevelopmental deficits. It has been demonstrated that the proposed method could be used for the determination of catecholamine presence in urine, with a promising prospect for use in the clinic for evaluating diseases relevant to these neurotransmitters.

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DISCLOSURES:

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in this article.

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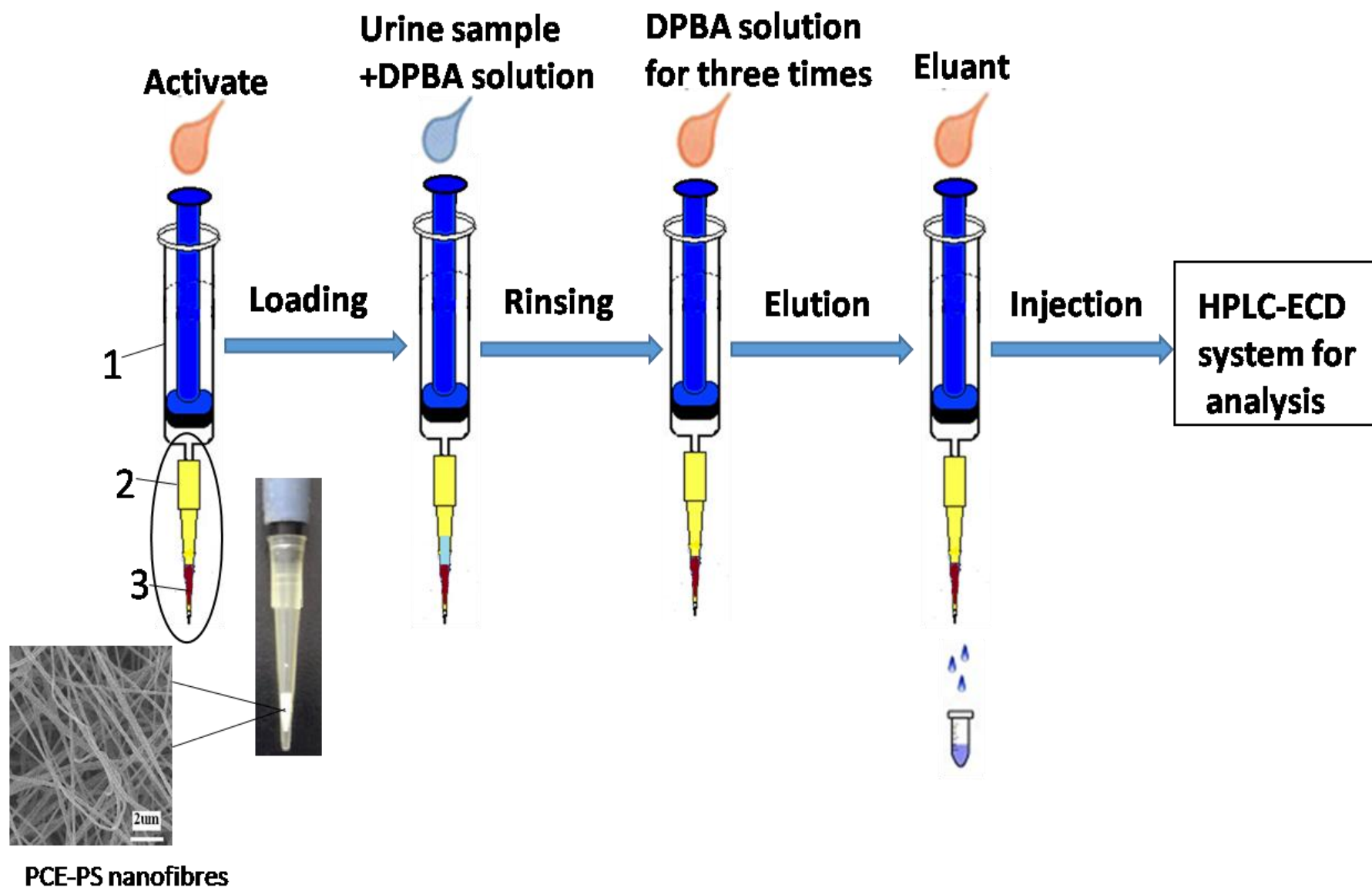


Figure 2

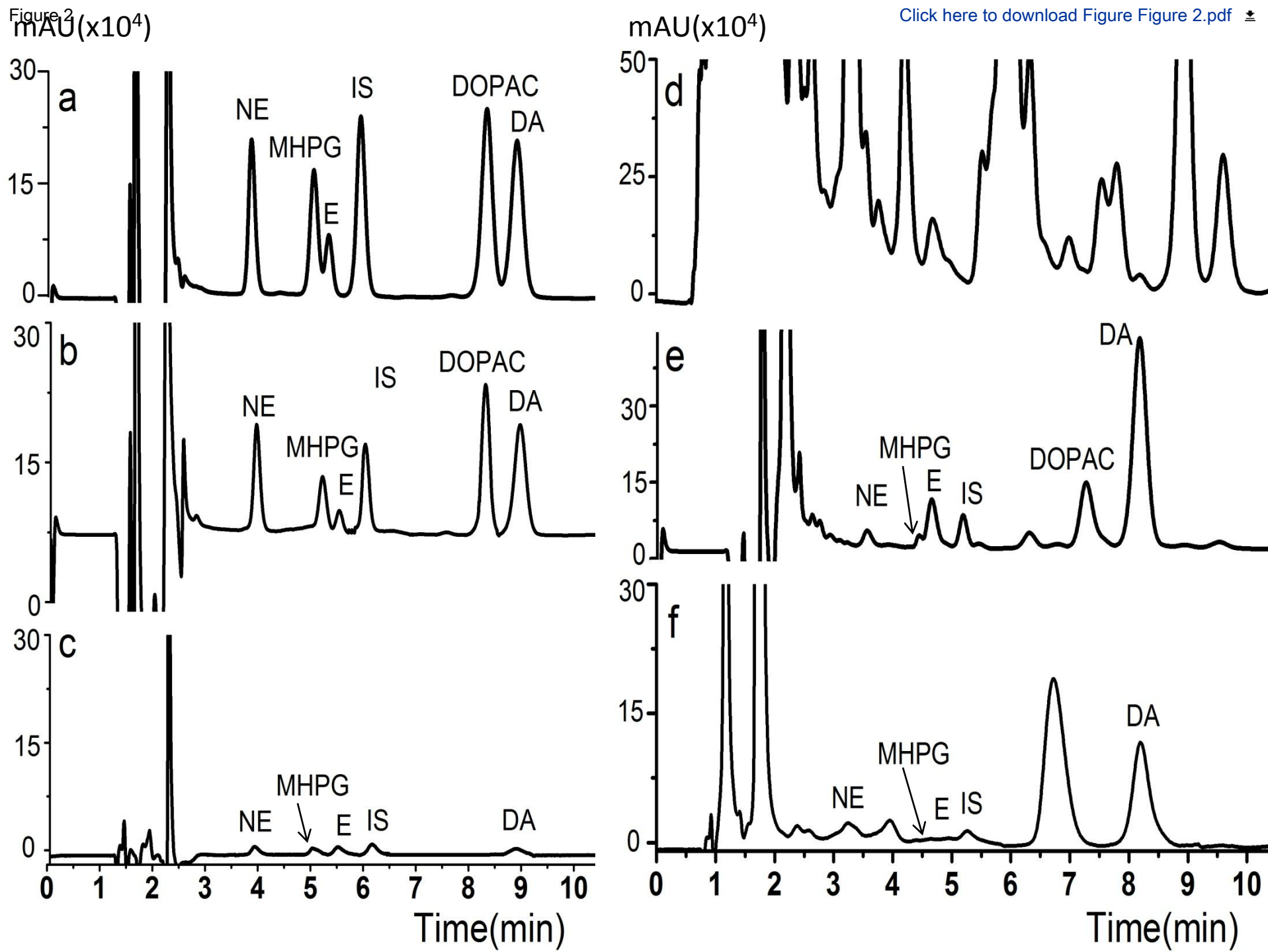
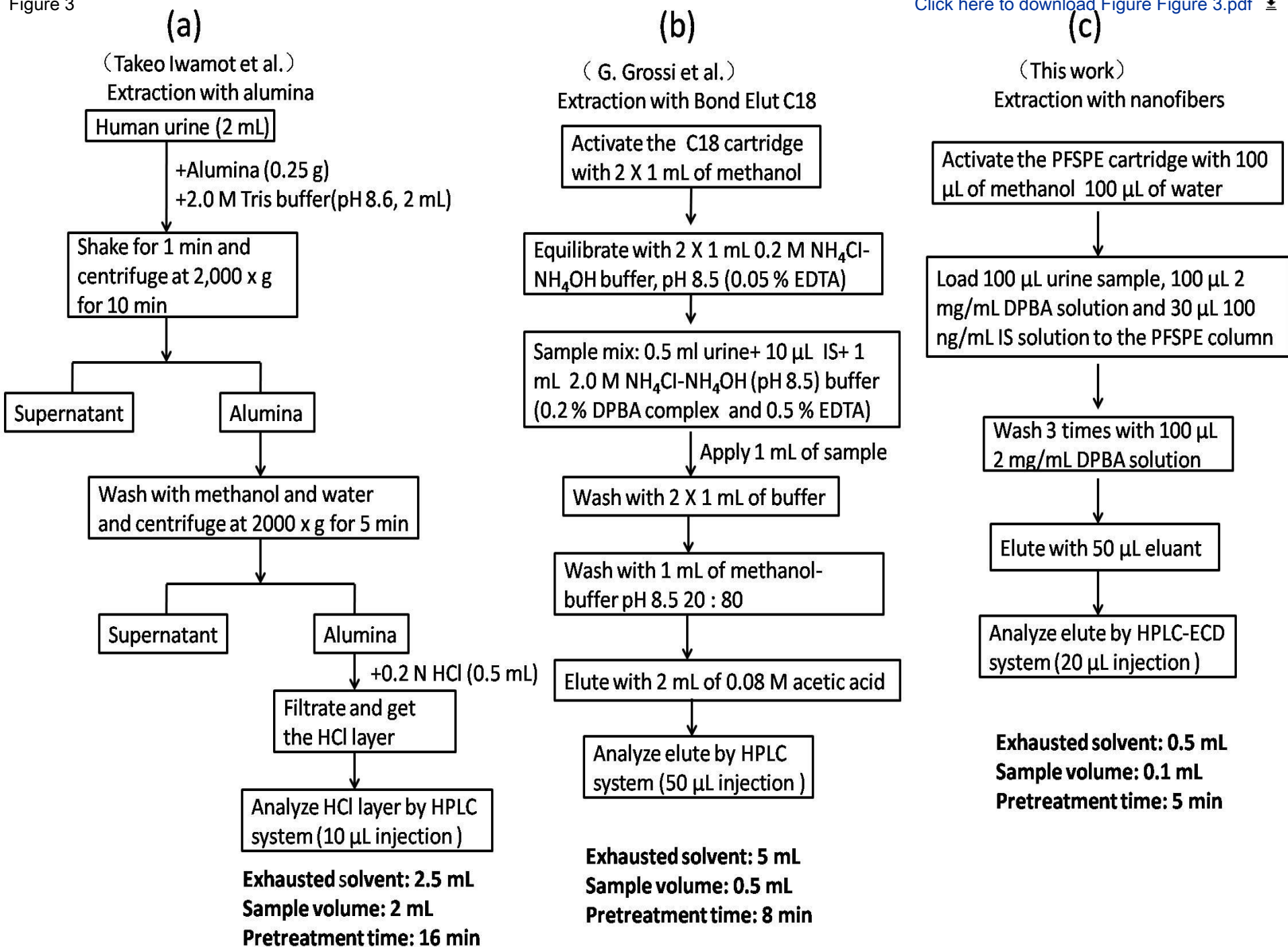


Figure 3



| | NE | MHPG | E | IS | DOPAC | DA |
|--------------------------|-----------|----------|-----------|----------|-----------|-----------|
| Linear range (ng/mL) | 1.5-400 | 1.5-200 | 1.5-100 | | 1.5-100 | 1.5-400 |
| R-squared values | 0.9945 | 0.995 | 0.9976 | | 0.9902 | 0.9954 |
| LOD (ng/mL) | 0.323 | 0.322 | 0.313 | 0.657 | 0.249 | 0.543 |
| LOQ (ng/mL) | 1.076 | 1.072 | 1.043 | 2.191 | 0.83 | 1.809 |
| Recovery ± RSD (%) (n=9) | 110.7±2.9 | 97.4±9.1 | 103.9±5.2 | 86.5±7.3 | 124.2±3.1 | 117.3±5.4 |
| Precision(RSD %) (n=9) | | | | | | |
| Intra-day | 4.5 | 4 | 2.7 | 4.8 | 3.3 | 4.7 |
| Inter-day | 4.1 | 8.1 | 2 | 6.3 | 3.2 | 5.9 |

NE, norepinephrine; MHPG, 3-Methoxy-4-hydroxyphenylglycol; E, epinephrine; IS, internal standard; DOPAC, 3, 4-Dihydroxyphenylacetic acid; DA, dopamine;

| Concentration (ng mL ⁻¹) | Control group (22) | High Risk Infant group (28) | <i>P</i> value |
|---|--------------------|--------------------------------|----------------|
| NE | 7.52±1.34 | 5.56±1.7 | 0.37 |
| MHPG | 1.4±0.2 | 14.8±3.6 | 0.001 |
| E | 24±15.8 | 20.9±5.87 | 0.841 |
| DOPAC | 106.36±30.1 | 72.12±18.07 | 0.312 |
| DA | 55.53±11.9 | 48.12±20.9 | 0.76 |

P<0.05***shows a significant difference between two groups

| Sample | Exhausted solvent (mL) | Sample volume (mL) | Pretreatment time (min) | Linear range | LOD |
|------------------|------------------------|--------------------|-------------------------|-------------------------------------|---|
| Urine | 0.5 | 0.05 | 10 | 2.0–200 ng/mL (NE,E,DA) | 0.2-0.5 ng/mL (NE,E,DA) |
| Urine | 19 | 0.7 | — | 47–167 µg/L (DA) | 166-500 nM (DA) |
| Urine | 1.3 | 0.01 | — | 0.5–1250 ng/mL (NE,E,DA,NMN,MN) | 0.5-2.5 ng/mL (NE,E,DA,NMN,MN) |
| Urine and plasma | 20 | 0.05 | 10 | 0.04-2.5 ng/mL (NE,E,DA) | 0.01-0.02 ng/mL (NE,E,DA) |
| Urine | <0.5 | 0.1 | 5 | 1.5-400 ng/mL (NE,MHPG,E,DOPAC, DA) | 0.249-0.543 ng/mL (NE,MHPG,E,DOPAC, DA) |

—, Undefined.
PBA, Phenylboronic acid

| Relative Recovery (%) | Evaporate and redissolve | Analytical method |
|----------------------------------|--------------------------|---|
| 88.5-94.5 (NE,E,DA) | No | HPLC-ECD (Chen <i>et al.</i> , 2016) |
| 98.3-101.1 (DA) | No | HPLC-UV (Piotr <i>et al.</i> , 2016) |
| 74.1–97.3 (NE,E,DA,NMN,MN) | No | LC-MS/MS (Li <i>et al.</i> , 2016) |
| 87.0-97.5 (NE,E,DA) | No | HPLC-UV (Mohammad <i>et al.</i> , 2016) |
| 97.4-124.2 (NE,MHPG,E,DOPAC, DA) | No | this work |

| Name of Material/ Equipment | Company | Catalog Number |
|---|------------------------------------|----------------|
| 200 µL pipette tip | | |
| PCE-PS nanofibers | | |
| steel rod (about 0.5 mm diameter) | | |
| gastight plastic syringe (5 ml) | | |
| methanol | Sinopharm Chemical ReagentCo., Ltd | 67-56-1 |
| diphenylborinic acid 2-aminoethyl ester(DPBA) | Sigma-Aldrich.Inc | A-106408 |
| norepinephrine(NE) | Sigma-Aldrich.Inc | A-9512 |
| 3-Methoxy-4-hydroxyphenylglycol(MHPG) | Sigma-Aldrich.Inc | H1377 |
| epinephrine(E) | Sigma-Aldrich.Inc | 100154-200503 |
| 3, 4-Dihydroxyphenylacetic acid(DOPAC) | Sigma-Aldrich.Inc | D-9128 |
| dopamine(DA) | Sigma-Aldrich.Inc | H-8502 |
| 3, 4-dihydroxybenzylamine hydrobromide(DHBA) | Sigma-Aldrich.Inc | 858781 |
| acetonitrile | Sigma-Aldrich.Inc | 75-05-8 |
| phosphoric acid | Sinopharm Chemical ReagentCo., Ltd | 7664-38-2 |
| uric acid | Sinopharm Chemical ReagentCo., Ltd | 69-93-2 |
| creatinine | Sinopharm Chemical ReagentCo., Ltd | 60-27-5 |
| trisodium citrate | Sinopharm Chemical ReagentCo., Ltd | 6132-04-3 |
| KCl | Sinopharm Chemical ReagentCo., Ltd | 7447-40-7 |
| NH ₄ Cl | Sinopharm Chemical ReagentCo., Ltd | 12125-02-9 |
| NaHCO ₃ | Sinopharm Chemical ReagentCo., Ltd | SWC0140326 |
| C ₂ Na ₂ O ₄ | Sinopharm Chemical ReagentCo., Ltd | 62-76-0 |
| NaSO ₄ | Sinopharm Chemical ReagentCo., Ltd | 7757-82-6 |
| disodium hydrogen phosphate | Sinopharm Chemical ReagentCo., Ltd | 10039-32-4 |
| urea | Sinopharm Chemical ReagentCo., Ltd | 57-13-6 |
| NaCl | Sinopharm Chemical ReagentCo., Ltd | 7647-14-5 |
| MgSO ₄ ·7H ₂ O | Sinopharm Chemical ReagentCo., Ltd | 10034-99-8 |
| CaCl ₂ | Sinopharm Chemical ReagentCo., Ltd | 10035-04-8 |
| HCl | Sinopharm Chemical ReagentCo., Ltd | 7647-01-0 |
| citric acid | Sinopharm Chemical ReagentCo., Ltd | 77-92-9 |
| EDTA disodium salt | Sinopharm Chemical ReagentCo., Ltd | 34124-14-6 |
| monometallic sodium orthophosphate | Sinopharm Chemical ReagentCo., Ltd | 7558-80-7 |
| 1-heptanesulfonic acid sodium salt | Sinopharm Chemical ReagentCo., Ltd | 22767-50-6 |

| | | |
|---|-------------------------------------|------------|
| sodium hydroxide | Sinopharm Chemical Reagent Co., Ltd | 1310-73-2 |
| phenylboronic acid column(PBA column) | Agilent | 12102018 |
| Inertsil® ODS-3 5 µm 4.6×150 mm column | Dikma | 5020-06731 |
| SHIMADZU SIL-20AC prominence AUTO SAMPLER | Shimadzu Corporation, Japan | SIL-20AC |
| SHIMADZU LC-20AD High Performance Liquid Chromatography | Shimadzu Corporation, Japan | LC-20AD |
| SHIMADZU L-ECD-60A electrochemical detector | Shimadzu Corporation, Japan | L-ECD-60A |
| ASAP 2020 Accelerated Surface Area and Porosimetry | Micromeritics, USA | |

Comments/Description

column to contain nanofibers

material for PFSPE extraction

fill the nanofibres into the column

compress solution into the end of the tip

complex reagent

analyte

analyte

analyte

analyte

analyte

interior label

eluriant and mobile phase

eluriant

artificial urine

artificial urine

artificial urine

artificial urine

artificial urine

artificial urine

artificial urine

artificial urine

artificial urine

artificial urine

artificial urine

artificial urine

artificial urine

artificial urine

artificial urine and mobile phase

mobile phase

artificial urine and mobile phase

mobile phase

mobile phase

PBA extraction

HPLC column for separation

auto injection for eluent

HPLC pump

detector for the analytes

surface and porosity analyzer



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| Article Title: | A Convenient Method for Catecholamines Neurotransmitters and Their Metabolites Extraction and Analysis with High-pressure Liquid Chromatography in Infants' Urine | | |
| Signature: |  | Date: | 19, April, 2017 |

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Editorial comments:

Please address the following concerns:

General:

1. While we appreciate the effort you have put in editing the language, parts of the manuscript are still difficult to understand. We have largely edited the Introduction (please review to insure your intended meaning is retained) and highlighted (in green) other areas of concern, although this is not intended to indicate every issue. Please edit and have a fluent English speaker review the manuscript (including paid services, if necessary).

Respond: Firstly, we are greatly appreciating your edition for the paper. All the highlighted part have been reviewed and modified by us (the green highlighting color has been removed from the paper while all the changing have been tagged).

2. Please ensure the manuscript is in American English (e.g., analyze instead of analyse, nanofiber instead of nanofibre).

Respond: The words you mentioned have all been modified.

3. Please ensure all Latin-derived phrases (et al., e.g., etc., i.e.) are italicized.

Respond: All the Latin-derived phrases (et al., e.g., etc., i.e.) are italicized in the paper (including references).

Figures and Tables:

1. Figure 2: Please ensure the arrows don't overlap 'MHPG.'

2. Figure 3: "Filtrate", not "Filtrated."

3. Please remove ® from all tables (including supplemental tables), and ensure all text is in the same font.

Respond: We have modified the Figures and tables as you required.

Protocol:

1. Note that the formatting of the protocol has been changed slightly to better fit JoVE's standards.

2. Please split up some of your longer steps so that each step contains 2-3 actions and no more than 4 sentences.

Respond: We have split up some steps into smaller ones as you required, including step 5.1 to 5.4 (from line 298-374)

3. Please ensure that most of the numbered steps are in the imperative tense, and that every step includes at least one sentence in the imperative tense.

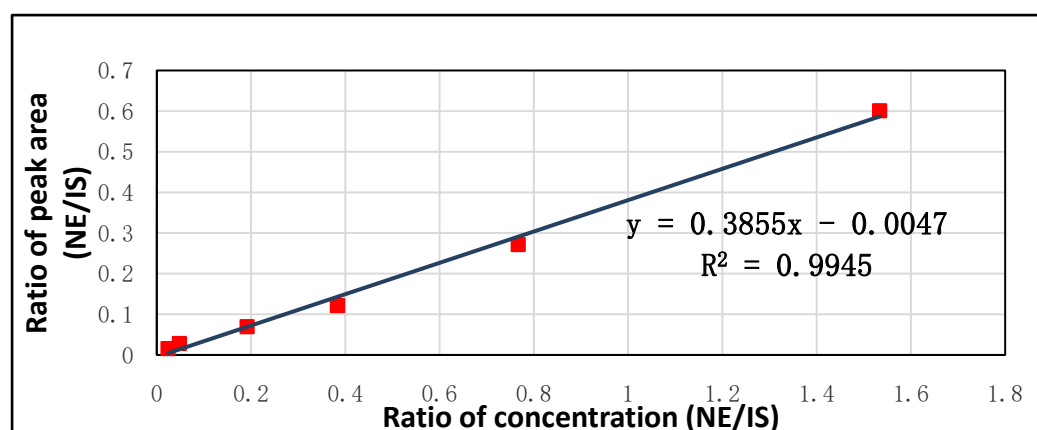
Respond: We have modified the Protocol as you required. Every step includes at least one sentence in the imperative tense.

Discussion:

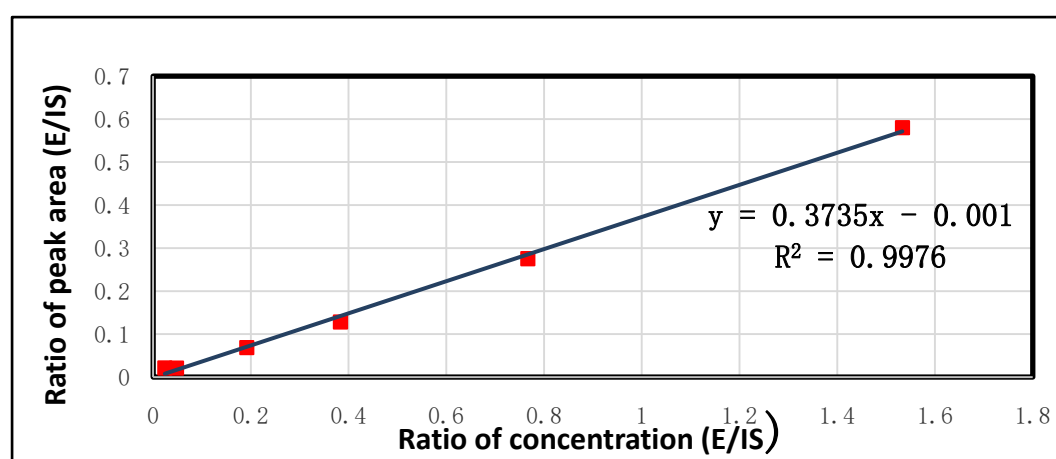
1. Please split the second paragraph into at least 2 smaller paragraphs

Respond: We have split the second paragraph into 2 smaller paragraphs.

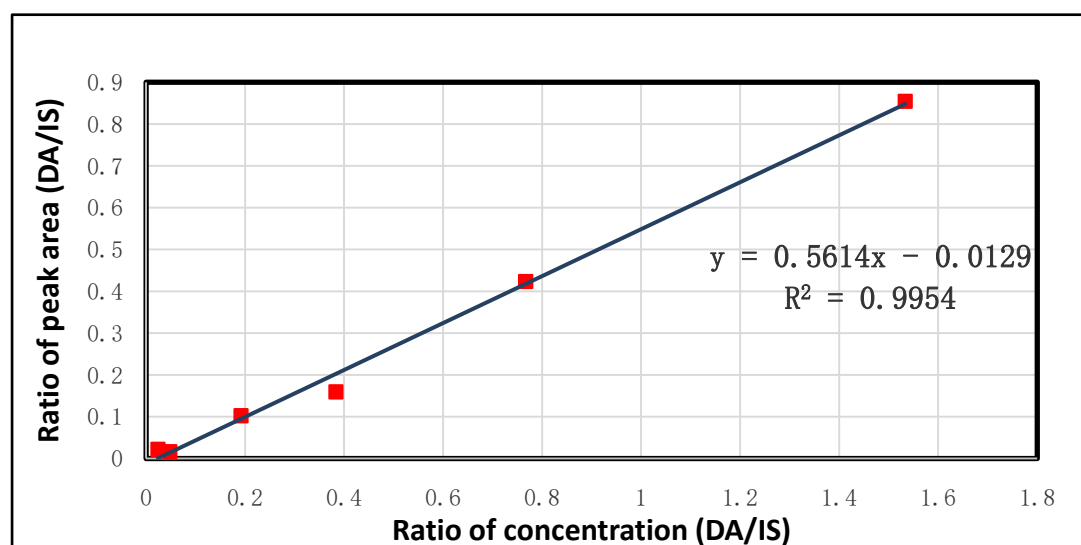
(a)



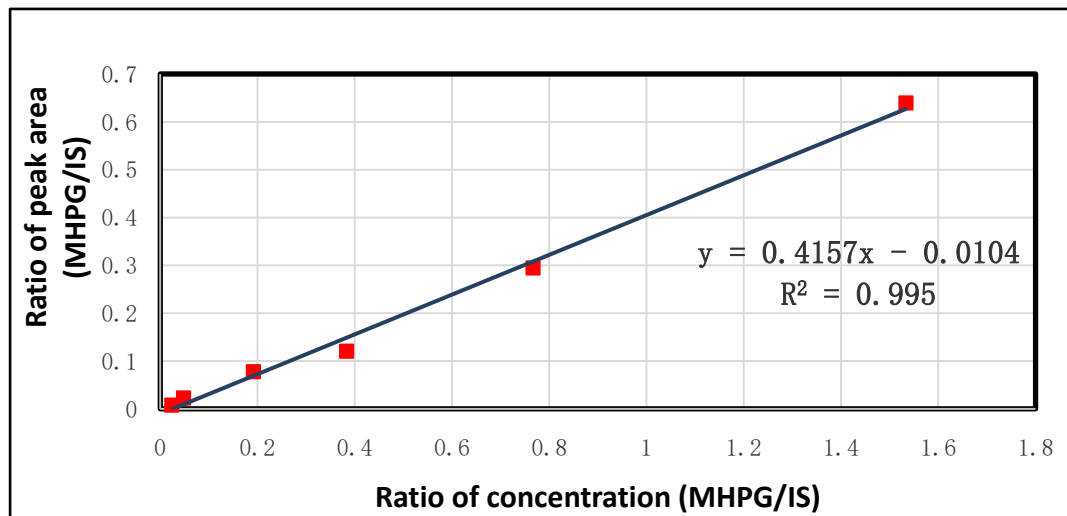
(b)



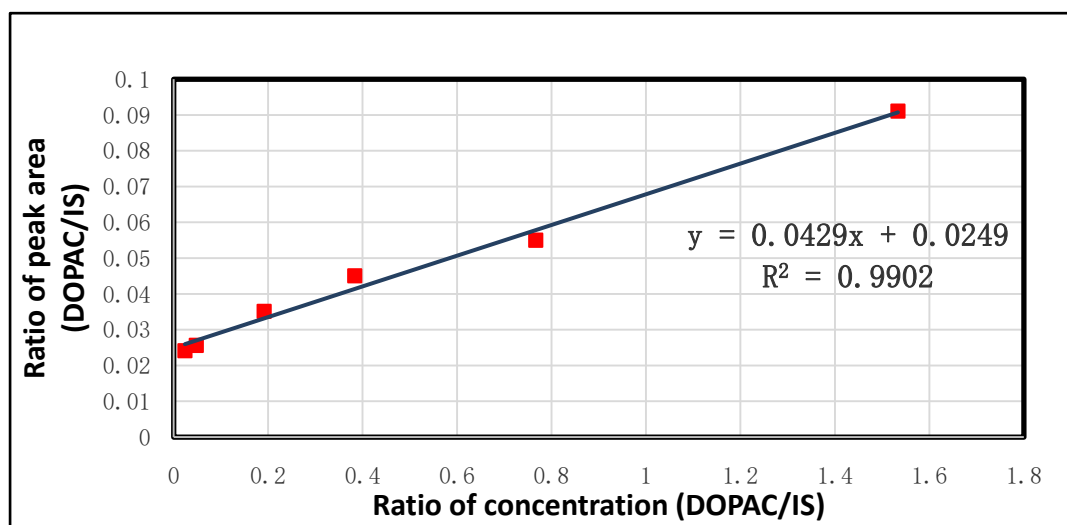
(c)



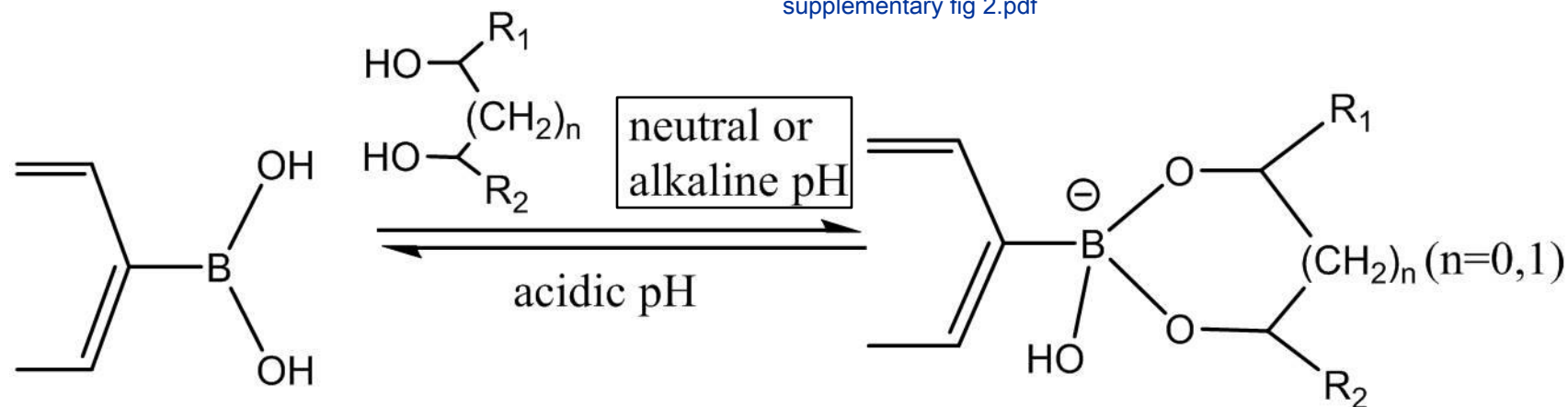
(d)



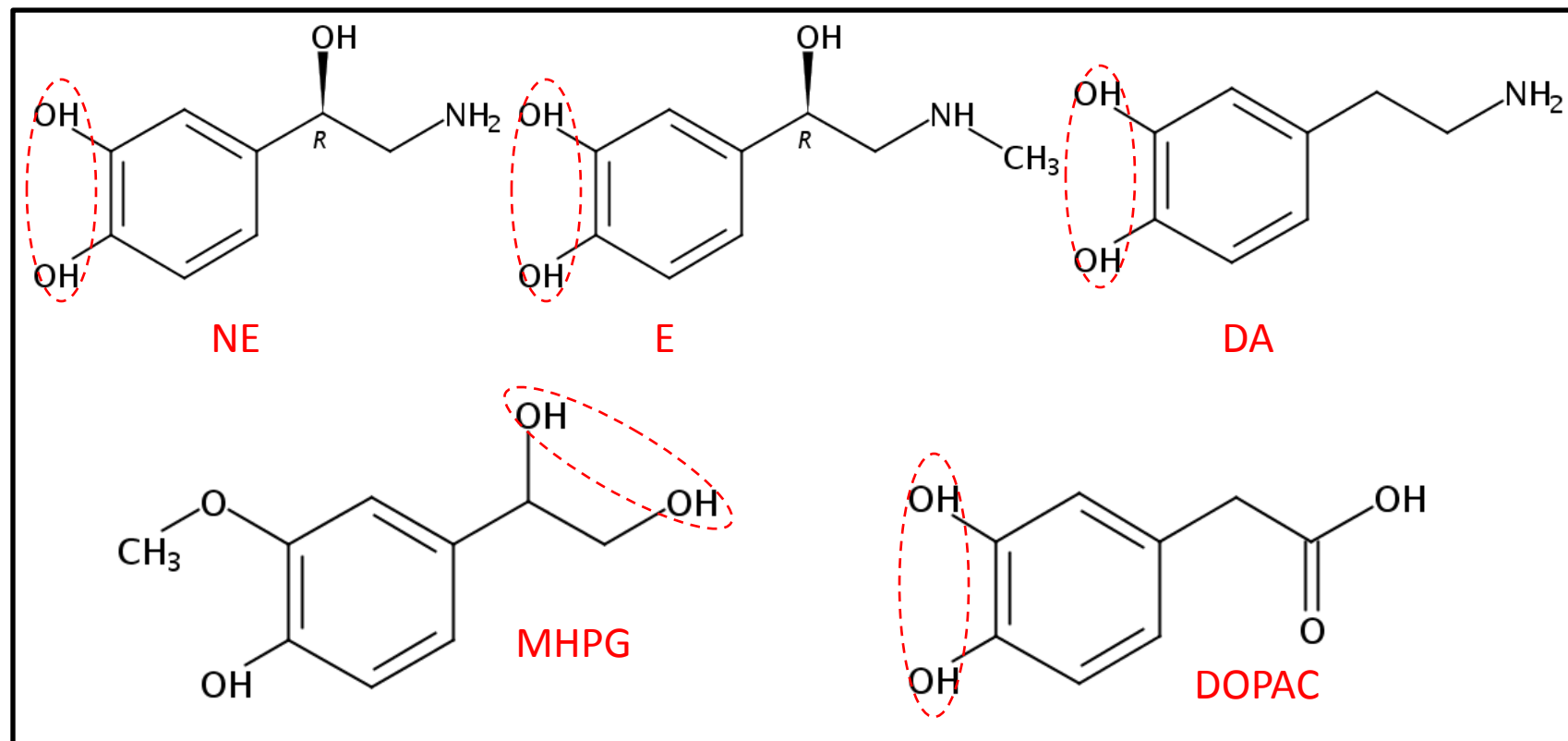
(e)



A



B



| | |
|------------------------------|--|
| Mobile phase | 4% acetonitrile and ionic species, citric acid 35 mmol/L, ethylene diamine tetra acetic acid (EDTA) disodium salt 0.25 mmol/L, monometallic sodium orthophosphate sodium acetate 45 mmol/L, 1-heptanesulfonic acid sodium salt 2 mmol/L, and add saturated sodium hydroxide solution to adjust pH 4.21 |
| Mobile phase flow rate | 1.0 mL/min |
| Column type | Inertsil ODS-3 5 µm 4.6×150 mm column, Dikma |
| Column length | 150mm |
| Column inner diameter | 4.6mm |
| Column temperature | 30 °C |
| Detector temperature | 30 °C |
| Voltage setting for analytes | 0.67 V |
| Injection volume | 20 µL |

| ng/mL | 1000 ng/mL standard, µL | artificial urine, µL | Dilution, fold |
|-------|-------------------------|----------------------|----------------|
| 100 | 50 | 450 | 10 |
| 50 | 25 | 475 | 20 |
| 25 | 12.5 | 487.5 | 40 |
| 12.5 | 6.25 | 493.8 | 80 |
| 3.125 | 1.5 | 498.5 | 320 |
| 1.5 | 0.8 | 499 | 640 |