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# Preparation of methacrylate-based monolith for capillary hydrophilic interaction chromatography and its application in determination of nucleosides in urine

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ABSTRACT

A novel poly(*N*-acryloyltris(hydroxymethyl)aminomethane-*co*-pentaerythritol triacrylate) (NAHAM-*co*-PETA) monolith was prepared in the 100 μm i.d. capillary and investigated for capillary liquid chromatography (*c*LC). The polymer monolith was synthesized by *in situ* polymerization of NAHAM and PETA in the presence of polyethylene glycol (PEG) in dimethyl sulfoxide (DMSO) as the porogen. The porous structure of monolith was optimized by changing the ratio of NAHAM to PETA, the molecular weight and amount of PEG. To evaluate the separation performance of the resultant polymer monolith, several groups of model compounds (including nucleosides, benzoic acids and anilines) were selected to perform *c*LC separation. Our results showed that these model compounds can be baseline separated on the resultant poly(NAHAM-*co*-PETA) monolithic column with the optimized mobile phases. The column efficiency was estimated to be 87,000 plates/m for acrylamide. In addition, this monolithic column was coupled with on-line solid-phase microextraction (SPME) for the analysis of four nucleosides (uridine, adenosine, cytidine, guanosine) in urine. The limit of detection of the proposed method was in the range from 40 to 52 ng/mL. The method reproducibility was obtained by evaluating the intra- and inter-day precisions with relative standard deviations (RSDs) less than 8.3% and 10.2%, respectively. Recoveries of the target analytes from spiked urine samples were ranged from 86.5% to 106.8%.



## 1. Introduction

Hydrophilic interaction chromatography (HILIC) has emerged as a complementary technique to reversed-phase liquid chromatography (RPLC) [1–3] due to its distinct merits in separation of polar compounds [4,5] and sensitivity enhancement for electrospray ionization mass spectrometry (ESI-MS) [6,7]. HILIC has been successfully used in the analysis of carbohydrates [7–9], peptides [10,11], proteins [12,13], natural product extracts [14,15], polar pharmaceuticals [16,17], and so forth.

Since organic polymer-based monoliths were initially introduced for capillary liquid chromatography (*c*LC) by Hjertén's group [18], porous polymeric monoliths are intensively employed as *c*LC stationary phase. Methacrylate-based monolith, one type of organic polymer-based monoliths, has been widely applied as separation media, due to its unique advantages such as excellent stability under extreme pH conditions and simple preparation process. A variety of methacrylate-based monoliths with hydroxyl [19], amino [20–23], amide [24,25], cyano [26], sulfonic [27–29], carboxylic [30] moieties have been prepared for *c*LC. However, the application of methacrylate-based monolithic columns for HILIC is hampered by the lack of commercially available polar monomers and the limited solubility of very polar monomers in common solvents [1,5].

Obviously, different from the hydrophilicity of silica-based stationary phase, the polymer skeleton bone of methacrylate-based monolith is hydrophobic. Hence, in order to circumvent the impediments, polar crosslinkers are adopted. For instance, Xie's group prepared a polar methacrylate-based monolithic column with pentaerythritol triacrylate (PETA) as the crosslinker [31]. The hydroxyl group of PETA can form hydrogen bond with polar analytes to provide sufficient hydrophilic retention. On the other hand, to improve hydrophilicity of methacrylate-based monolith, monomers with charged groups are frequently adopted. Jiang and co-workers reported several methacrylate-based monolith for capillary HILIC (*c*HILIC), adopting polar monomers with zwitterionic groups [5,32,33]. On these monolithic columns, typical HILIC retention was observed in the separation of polar analytes. However, when the ionization of functional groups can be influenced by the mobile phase pH, the selectivity of charged analytes depends on the ionic strength and mobile phase pH. So far as we know, a few attempts have been reported for the preparation of methacrylate-based monolithic columns as the polar stationary phases, especially pH- and salt-independent polar methacrylate-based monoliths [32–34]. Efforts in preparation of novel polar stationary phases

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for highly efficient separation of polar compounds can benefit the promotion of *c*HILIC.

In current study, a novel polar methacrylate-based monolith was prepared. The structure of monolith was optimized by changing the ratio of the functional monomer (*N*-acryloyltris(hydroxymethyl)aminomethane, NAHAM) to the crosslinker (PETA), the amount of dimethyl sulfoxide (DMSO), as well as the molecular weight and amount of PEG. The monolithic column obtained under optimized conditions had homogeneous and continuous column bed, good permeability, highly mechanical stability and extraordinary column efficiency (up to 87,000 plates/m). Moreover, as *c*HILIC stationary phase, the performance of monolithic column was assessed in detail and the resulting monolith showed good selectivity in separation of nucleosides, benzoic acid derivatives and anilines. Moreover, due to hydrophilic interaction between analytes and hydroxyl groups of monoliths, the separations of different kinds compounds are pH- and ionic strength independent. This feature can benefit the development of on-line solid phase microextraction-capillary liquid chromatography (online SPME-*c*LC) coupled with certain extraction column, which requires the eluent containing high ACN content, high salt concentration under acidic or basic conditions. Then, since four nucleosides (uridine, adenosine, cytidine, guanosine) were effectively extracted by poly(3-acrylamidophenylboronic acid-*co*-ethylene dimethacrylate) (AAPBA-*co*-EDMA) monolith [35], a high sensitive on-line SPME-*c*LC was established for analysis of nucleosides in urine samples. The relevant methodological investigation and the application in the nucleosides in urine samples were also studied.

## 2. Materials and methods

### 2.1. Chemicals and buffers

NAHAM (98% pure), PETA (98% pure), 3-acrylamidophenylboronic acid (AABPA, 98% pure) and ethylene dimethacrylate (EDMA, 98% pure) were purchased from Acros (NJ, USA). Azobisisobutyronitrile (AIBN), PEG with molecular weights from 1000 to 10,000, and DMSO were all purchased from Shanghai Chemical Reagent Corporation (Shanghai, China). 3-(triethoxysilyl) propyl methacrylate was purchased from Wuhan University Silicone New Material (Wuhan, China). HPLC-grade methanol and acetonitrile (ACN) were obtained from TEDIA Company (Ohio, USA). Ammonium hydroxide solution (25 wt%; LC–MS grade) was supplied by Sigma–Aldrich (St. Louis, MO, USA). The water used throughout all experiments was purified using a Milli-Q water purifier (Millipore, Bradford, USA). The fused-silica capillaries with 530 μm or 100 μm i.d. were purchased from Yongnian Optic Fiber Plant (Hebei, China).

Thymidine, uridine, adenosine and cytidine and guanosine were purchased from Shanghai Kayon Biological Technology (Shanghai, China). Thiourea, toluene, anilines, benzoic acids, formic acid (FA) and other chemicals of analytical grade used in the experiment were purchased from Shanghai Chemical (Shanghai, China). The standard solution of 1.0 mg/mL for each analyte was prepared in methanol and stored at −4 °C in the dark. With these standard solutions, the sample solutions were spiked to the desired concentration for experiments.

### 2.2. Preparation of urine samples

Urine samples of four tumor patients and two healthy volunteers were collected from the Zhongnan Hospital (Wuhan, China). All urine samples were kept frozen before use.

$NaHCO_3$ (0.084 g) was added in 1.0 mL urine, and then the urine sample was diluted with 4 mL ACN. After mixed with a vortex mixer, the sample was centrifuged at −4 °C for 5.0 min at 9990 g (Anting Scientific Instrument, Shanghai, China). Then the supernatant was filtered through a 0.22 μm micro-filter prior to on-line SPME-*c*LC analysis.

### 2.3. Preparation of the monoliths

The poly(NAHAM-*co*-PETA) monoliths were prepared by one-step thermally initiated polymerization method inside a fused silica capillary, whose inner surface was derivatized with 3-(triethoxysilyl) propyl methacrylate [35] and then dried with nitrogen gas. Firstly, porogen PEG was dissolved in DMSO (co-porogen) and thoroughly mixed to ensure that the solution was completely homogeneous. Subsequently, monomer NAHAM and crosslinker PETA were added with the ratios described in Table 1. The mixture was then briefly shaken before adding the initiator AIBN (1% w/w with respect to monomers). The polymerization mixture was completely mixed by vortexing and ultrasonication to form a homogeneous solution after adding AIBN. Then the resulting solution was filled into the capillary to 30 cm length. Special care was taken to ascertain that no air bubbles were trapped in the capillary. Both ends of the capillary were sealed by silicon rubber for polymerization at 60 °C for 12 h (Fig. 1). Finally, the capillary was washed with methanol to remove residual reagents.

Poly(NAHAM-*co*-PETA) monoliths were synthesized with PEG of different molecular weights as porogen (Table 1) in Eppendorf vial for specific surface area measurement. After polymerization, the polymers were cut into small cubic pieces and submersed in water/methanol (50/50, v/v) for at least 4 h to remove the PEG and non-reacted chemicals [36]. The washing step was repeated three times follow by drying in an oven at 100 °C. And the resulting samples were kept in a desiccator before characterization.

For on-line SPME, poly(AAPBA-*co*-EDMA) monolith was prepared according to previous report [35]. Briefly, the polymerization mixture was composed of 30 mg AAPBA, 70 mg EDMA, 265 mg methanol, 35 mg PEG-10,000 and 1 mg AIBN. Upon mixing, the homogeneous polymerization mixture was degassed by ultrasonication for 5 min, then filled into 530 mm i.d. fused-silica capillaries modified with 3-(triethoxysilyl)propyl methacrylate. Monoliths were fabricated by keeping the capillaries at 60 °C for 12 h followed by flushing with methanol to remove residual monomers and porogens.

### 2.4. Instrument and analytical conditions

The specific surface area of poly(NAHAM-*co*-PETA) monoliths were measured by nitrogen adsorption–desorption experiments with JW-BK surface area and pore size analyzer (JWGB Sci & Tech Co., Ltd., Beijing, China). Before measurement, the monolithic cubic pieces were evacuated in vacuum, and heated at 393 K for 3 h to remove the physically adsorbed substances. Specific surface area values were estimated by the Brunauer–Emmett–Teller (BET) equation at $P/P_0$ between 0.05 and 0.2 [37].

The microscopic morphology of the monoliths was examined using QUANTA-200 scanning electron microscopy (FEI, Eindhoven, Netherlands). Prior to measurement, the monolithic capillary samples were cut into 2 mm long pieces, placed on an aluminum stub and then sputter-coated with gold.

Back pressure and permeability of the monoliths were measured using a Shimadzu (Kyoto, Japan) LC-20A nanopump in the constant flow mode. ACN was pumped through the 30 cm-long capillaries with different flow rate ranging from 100 to 1300 nL/min. The back pressure was recorded after it stabilized.

**Table 1**
Optimization on monolithic column preparation.[a]

| Column | NAHAM (%, w/w$_{total}$) | PETA (%, w/w$_{total}$) | PEG MW | PEG (%, w/w$_{total}$) | State of column | Backpressure[b] (MPa) | Surface area (m$^2$/g) |
|---|---|---|---|---|---|---|---|
| 1 | 2.22 | 20.00 | 6000 | 20 | Homogeneous | 9.5 | – |
| 2 | 3.33 | 18.89 | | | Homogeneous | 6.4 | 66 |
| 3 | 4.44 | 17.78 | | | Slack, slight detached | 4.3 | – |
| 4 | 5.56 | 16.67 | | | Slack, slight detached | 2.8 | – |
| 5 | 3.33 | 18.89 | 1000 | 20 | Sol state | >22 | 120 |
| 6 | | | 2000 | | Homogeneous | 13.5 | 112 |
| 7 | | | 4000 | | Homogeneous | 11.4 | 95 |
| 8 | | | 10,000 | | Slack, slight detached | 3.8 | 51 |
| 9 | 3.66 | 20.73 | 6000 | 12.20 | Homogeneous | >22 | 106 |
| 10 | 3.49 | 19.77 | | 16.28 | Homogeneous | 9.8 | 88 |
| 11 | 3.19 | 18.09 | | 23.40 | Slack, slight detached | 4.9 | 56 |
| 12 | 3.06 | 17.35 | | 26.53 | Slack, slight detached | 3.2 | 37 |

[a] The amount of DMSO was 260 mg.
[b] Flushed with methanol; flow rate, 2 μL/min; column length, 10 cm.

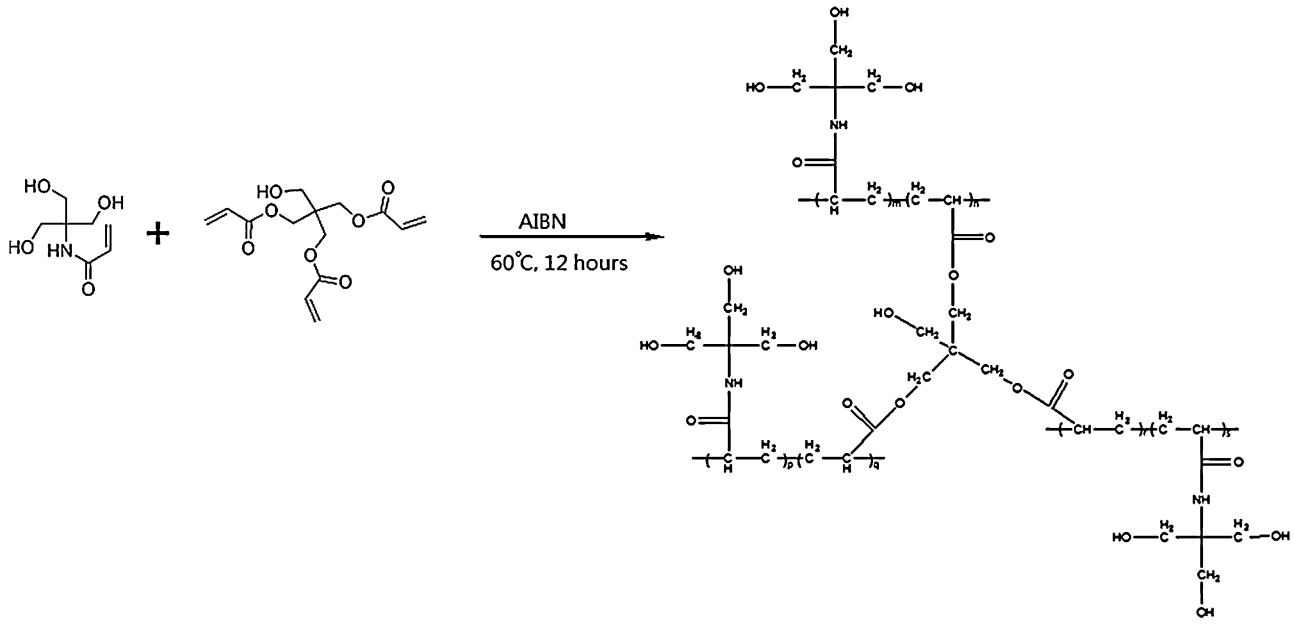



**Fig. 1.** Reaction scheme of poly(NAHAM-*co*-PETA) monolith.

Permeability ($K$, m$^2$) was calculated according to Darcy's law by the following equation [38,39]:

$$K = \frac{u\eta L}{\Delta P} \quad (1)$$

where $u$ (m/s) is the linear velocity of mobile phase, $\eta$ is the viscosity of the mobile phase (it is $0.60 \times 10^{-3}$ Pa·s at 20 °C in current study), $L$ is the length of the monolithic column ($m$) and $\Delta P$ is the pressure drop across the monolithic column (Pa).

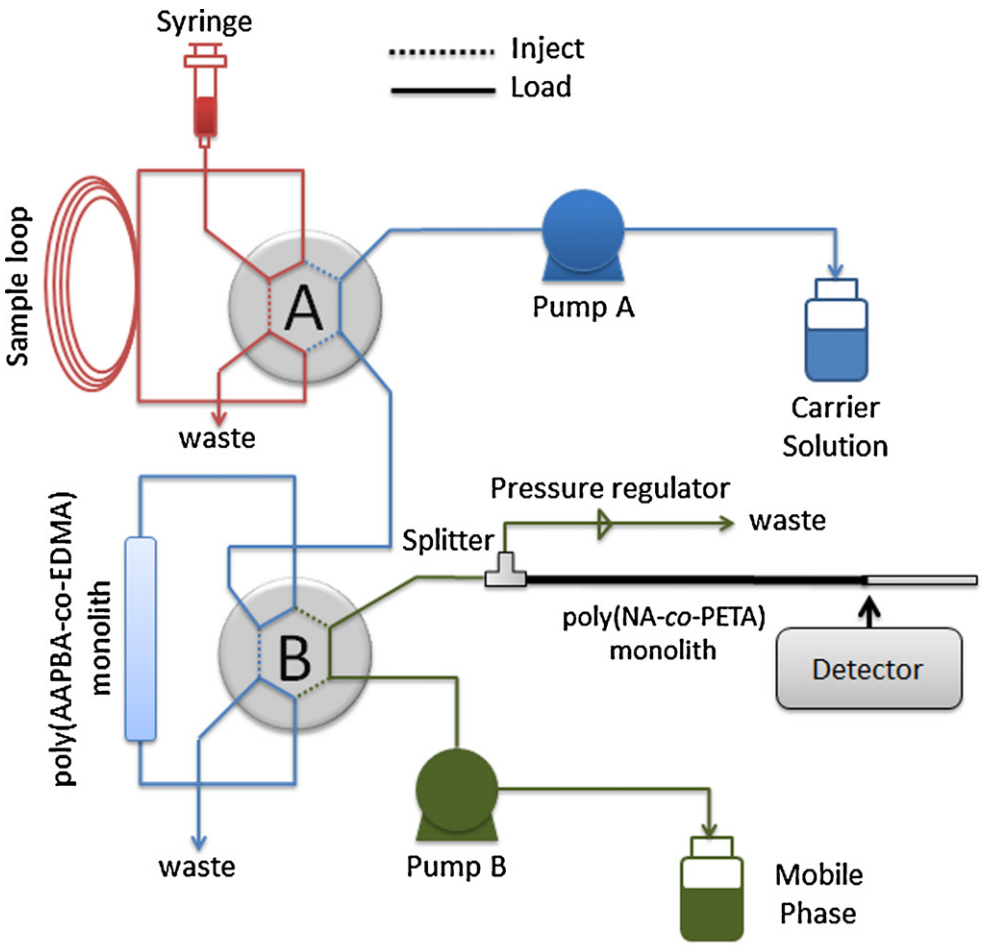
All *c*LC experiments were performed on a Unimicro Technologies Trisep™ 2010 *c*LC system (Shanghai, China) consisting of two HPLC pumps and GL Sciences MU 701 UV–vis detector (Tokyo, Japan). The Trisep HPLC pumps were also used to condition monolithic columns with mobile phase and drive out bubbles from capillaries. After connecting to the *c*LC system, the poly(NAHAM-*co*-PETA) monolith was conditioned with the mobile phase of water/ACN (50/50, v/v) under constant pressure of 10 MPa for 30 min. The chromatograms of benzoic acids were recorded at a wavelength of 214 nm, and 254 nm for other analytes.

On-line extraction and analysis was performed on a system set up in our laboratory (Fig. 2) [40,41], consisting of two microflow pumps (pumps A and B were from Unimicro Technologies, Shanghai, China), a high pressure six-way switching valve module (valve A and B, Shiseido, Tokyo, Japan) and a PEEK sample loop. As shown in Fig. 2, the sample was firstly loaded on the sample loop. While the on-line extraction program started, the valve A was switched to inject position, and the sample was driven by carrier solution (ACN/$H_2O$ (v/v, 80/20, adjust pH to 10.2 by adding ammonium hydroxide)) at 0.1 mL/min for 8 min into the poly(AAPBA-*co*-EDMA) monolith. After washing with carrier solution for 3 min at the same flow rate, the valve B was switched and the poly(AAPBA-*co*-EDMA) monolith was eluted with mobile phase (ACN/$H_2O$ (v/v, 98/2, adjust pH to 3.5 by adding FA)). After splitting in the three-port interface, the desorbed analytes were transferred to the inlet of analytical capillary under constant pressure controlled by a backpressure regulator.

## 3. Results and discussion

### 3.1. Preparation of poly(NAHAM-co-PETA) monolithic column

To obtain excellent mechanical stability and achieve high column efficiency, we optimized the porous structure of the poly(NAHAM-*co*-PETA) monolith. Several parameters can influence the porous structure of the monolithic column including the ratio of monomer over crosslinker, the molecular weight and amount of PEG [42,43].




**Time program for on-line SPME and *c*LC**

| Time | Process | Valve Position | |
|---|---|---|---|
| | | Valve A | Valve B |
| | Sample loading | Load | Load |
| 0'00'' | Sample injection/ Extraction Loading | Inject | Load |
| 8'00'' | Washing | | |
| 11'00'' | Elution | Inject | Inject |
| 11'20'' | Separation | Inject | Load |
| 26'20'' | Program completion | Load | Load |

**Fig. 2.** Construction and procedure of on-line SPME-cLC.

An alteration in the ratio of monomer to crosslinker may change the porosity, rigidity, and homogeneity of the monolith [43,44]. The ratios of monomer (NAHAM) to crosslinker (PETA) were optimized by increasing the NAHAM content from 2.22% to 5.56% ($w/w_{total}$) while the total weight of the monomer and crosslinker was kept constant (Table 1, columns 1–4). Using the homogeneity and backpressure as assessment criteria, we found that the monolith was detached when the NAHAM content increased to 4.44% and 5.56% ($w/w_{total}$) (Table 1, columns 3, 4), which can be explained that the decreased content of crosslinker was not sufficient to form firm bond to the inner wall of capillary. Since homogeneous monoliths could be obtained with the NAHAM content of 2.22% and 3.33% ($w/w_{total}$), NAHAM content was kept at 3.33% ($w/w_{total}$) for further experiment due to more functional groups and relatively low backpressure.

In order to find out the appropriate permeability and specific surface area as large as possible, five types of PEG with molecular weight from 1000 to 10,000 were investigated (Table 1,

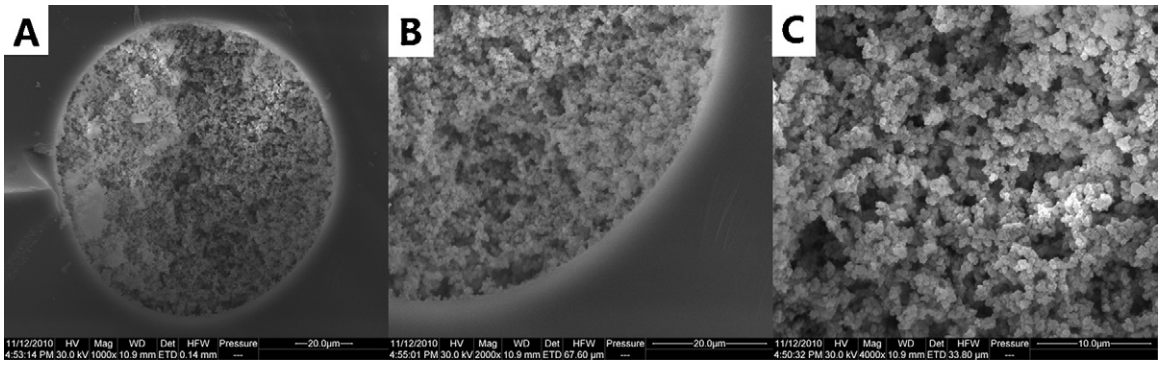



**Fig. 3.** Scanning electron microscope images of the cross section of the poly(NAHAM-*co*-PETA) monolith under optimized preparation conditions (column 2 in Table 1). (a) Wide-view and (b) and (c) close-up-view.

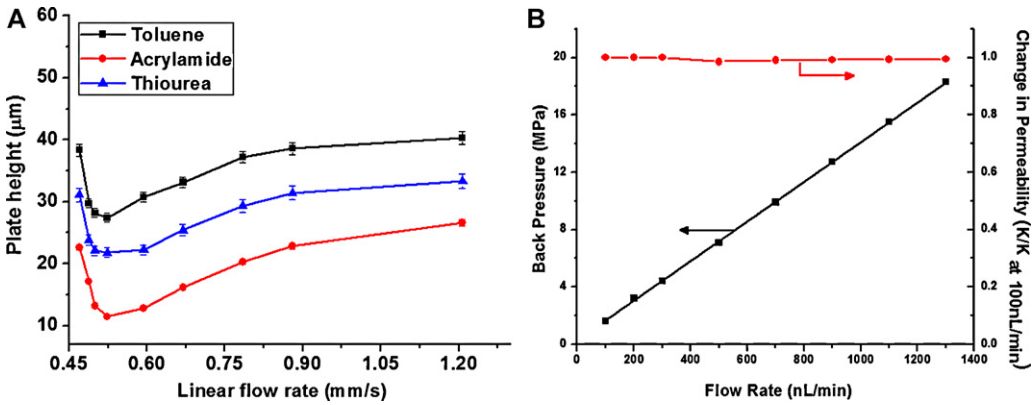



**Fig. 4.** Van Deemter plot of the height equivalent to a theoretical plate as a function of flow rate (A) and the effect of flow rate on back-pressure and variation of column permeability (B). Experimental conditions: monolithic capillary column, 100 μm i.d. × 30 cm; mobile phase, ACN/$H_2O$ (95/5, v/v); injection volume, 20 nL; UV detection wavelength, 254 nm.

columns 2, 5–8). We found the backpressure of the monoliths decreased from higher than 22 MPa to 6.4 MPa when the molecular weight of PEG increased from 1000 to 6000 (Table 1, column 2, 5–7). The monolith was detached when the molecular weight of PEG increased to 10,000 (column 8). In addition, the specific surface areas of the monoliths decreased monotonously from 120 to 51 with the increased molecular weight of PEG, which indicated that increasing the chain length of PEG can resulted in larger through-pore and smaller surface area. According to Courtois' report [36], the behavior of PEG with different chain lengths is similar in DMSO, and increasing the chain length only caused a solvated system with higher sterical hindrance. As a porogen, PEG could occupy certain space in monoliths. This space will become through-pores after removing porogen PEG. Therefore, PEG with long chain can lead to larger through-pores and smaller surface area. Considering that monolith should possess large surface area as well as appropriate permeability to provide sufficient chromatographic retention, we chosen PEG-6000 for further experiment.

Given the limited solubility of the PEG-6000 in DMSO, we evaluated the effect of PEG-6000 content from 12.20% to 26.53% ($w/w_{total}$) on the porous structure of poly(NAHAM-*co*-PETA) monolithic column (Table 1, columns 2, 9–12). The results showed that the backpressure of the monoliths decreased to 3.2 MPa when the amount of PEG-6000 increased to 26.53% ($w/w_{total}$) (Table 1, columns 2, 9–12). In general, addition of porogen can result in phase segregation in solution, leading to larger flow through channels [45,46]. Although the high percent PEG-6000 can improve the permeability of the monolith, it was observed that prepared monoliths were detached when the PEG content increased to 23.40% and 26.53% ($w/w_{total}$) (Table 1, columns 11, 12). In addition, the specific surface area of monoliths decreased from 106 to 37 $m^2/g$ when the PEG-6000 content increased from 12.20% and 26.53% ($w/w_{total}$). Consequently, 20% ($w/w_{total}$) PEG-6000 was employed in the following studies.

### 3.2. Characterization of the monolithic column

The morphology of the optimized poly(NAHAM-*co*-PETA) monolithic column (Table 1, column 2) was examined by SEM. Fig. 3A and B shows that the monolith was well attached to the inner wall of the capillary. In Fig. 3C, it can be observed that the microglobules were interconnected to form large clusters that yielded continuous microglobules, which resulted in a polymer-based monolithic matrix with uniform through-pores of approximate 2 μm.

The column efficiency of the poly(NAHAM-*co*-PETA) monolith was evaluated by changing the flow rate from 0.45 to 1.2 mm/s. The effect of the flow velocity (calculated from the retention time of toluene at different flow rate) on the plate height was examined by using thiourea, toluene and acrylamide (Fig. 4A). The results showed that the plate height first decreased when the linear velocity increased from 0.45 to 0.50 mm/s. However, a almost linear increase of the plate height was observed when the linear velocity increased from 0.50 to 1.2 mm/s. This Van Deemter curves showed the lowest plate height was approximated 11.5 μm for acrylamide with a flow velocity of 0.5 mm/s. The result demonstrated a typical characteristic of monolithic column that the column efficiency decreased slightly with the increasing flow rate from 0.50 to 1.2 mm/s.

The stability of this monolithic column was also tested with an increase of the mobile phase flow rate from 100 to 1300 nL/min. As shown in Fig. 4B, the back-pressure linearly increased with the increased flow rate. The effect of mobile phase flow rate on the permeability is also shown in Fig. 4B. The permeability was around $21.2 \times 10^{-13}$ m$^2$ with the increased flow rate, demonstrating that the column bed and porous structure of poly (NAHAM-*co*-PETA) monolith were stable under high back pressure (18 MPa).

### 3.3. Evaluation of poly(NAHAM-co-PETA)monolithic capillary for cLC

#### 3.3.1. HILIC behavior

A mixture of three compounds with different polarities, toluene, thioruea and acrylamide, were used to investigate the HILIC retention mechanism on this monolithic column. As shown in Fig. 5A, strong retention of polar compounds, thioruea and acrylamide, was observed with increased ACN content (>80%, v/v). As expected, the retention times of these two compounds were significantly increased with a raise in the ACN content from 80% to 95%, while there was no obvious change of the retention time of the non-polar toluene in this situation. This results demonstrated a typical HILIC retention mechanism on this poly(NAHAM-*co*-PETA) monolithic column, which was consistent with previous study [5].

#### 3.3.2. Separation of neutral polar compounds

The separations of five nucleosides (thymidine, uridine, adenosine, cytidine and guanosine) were investigated on the resultant HILIC monolith. As shown in Fig. 5B, the retention time of these five nucleosides increased with the increase of ACN content from 85% to 96%, which agreed well with the retention mechanism in HILIC. Generally, as the ACN content in mobile phase increased, the hydrophilic interaction was strengthened, which resulted in the extended retention time of the analytes (Fig. 5C).

#### 3.3.3. Separation of acidic compounds

The separations of seven benzoic acid derivatives were evaluated in different mobile phase with pH ranging from 3.3 to 7.5 (Fig. 6A). The results showed that the retention of these seven benzoic acid derivatives responded torpidly to the change of pH (Fig. 6A). Although the change of mobile phase pH can influence the degree of ionization of benzoic acid derivatives, their retentions were almost not changed due to little anion-exchange interaction between acidic analytes and the stationary phase.

The effect of ionic strength on rentention factor $k$ of benzoic acids was investigated at various concentrations of ammonium formate from 10 to 40 mM at pH 7.2 containing 85% (v/v) ACN (Fig. 6B). Generally, elution capability of mobile phase in ion-exchange chromatographic mechanism depended on the ionic strength of mobile phase. As the buffer salt concentration of mobile phase increased, the strength of the ion-exchange interaction decreased, which made the retention time of analytes shorter. As seen in Fig. 6B, the $k$ slightly decreased with the increase of ammonium formate concentration, which exhibited weak ion-exchange interaction between benzoic acids and the monolithic column.

Moreover, the content of ACN in the mobile phase has significant influence on the resolution and selectivity of benzoic acids, and hydrophilic interactions were apparently weakened by increasing the ACN content. The $k$ of seven benzoic acids decreased with the decrease of ACN content in the mobile phase from 95% to 80% (v/v) (Fig. 6C). The results indicated that a good separation of these solutes can be achieved just by changing the ACN content.

#### 3.3.4. Separation of basic compounds

Similar to the effect of mobile phase pH on the separation of benzoic acids, the retention times of the five anilines were slightly

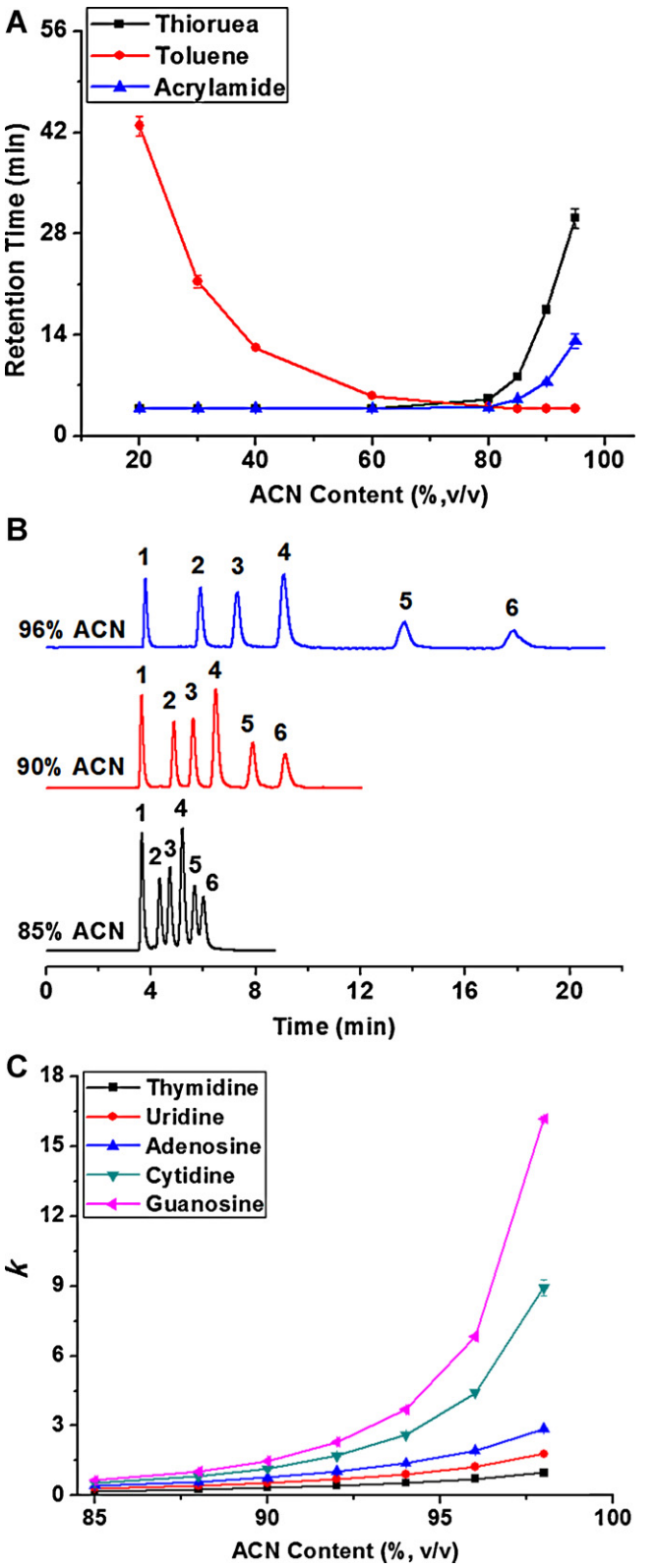



**Fig. 5.** Relationship between retention time and acetonitrile concentration on poly(NAHAM-*co*-PETA) monolithic column (A), chromatograms of nucleosides at different mobile phase pH (B) and (C) the retention factors of nucleosides with different ACN content. Experimental conditions: monolithic capillary column, 100 μm i.d. × 30 cm; flow rate, 400 nL/min; injection volume, 20 nL; mobile phase, ACN/$H_2O$ (v/v), pH 7.0; UV detection wavelength, 254 nm. Peaks: 1, toluene; 2, thymidine; 3, uridine; 4, adenosine; 5, cytidine; 6, guanosine.

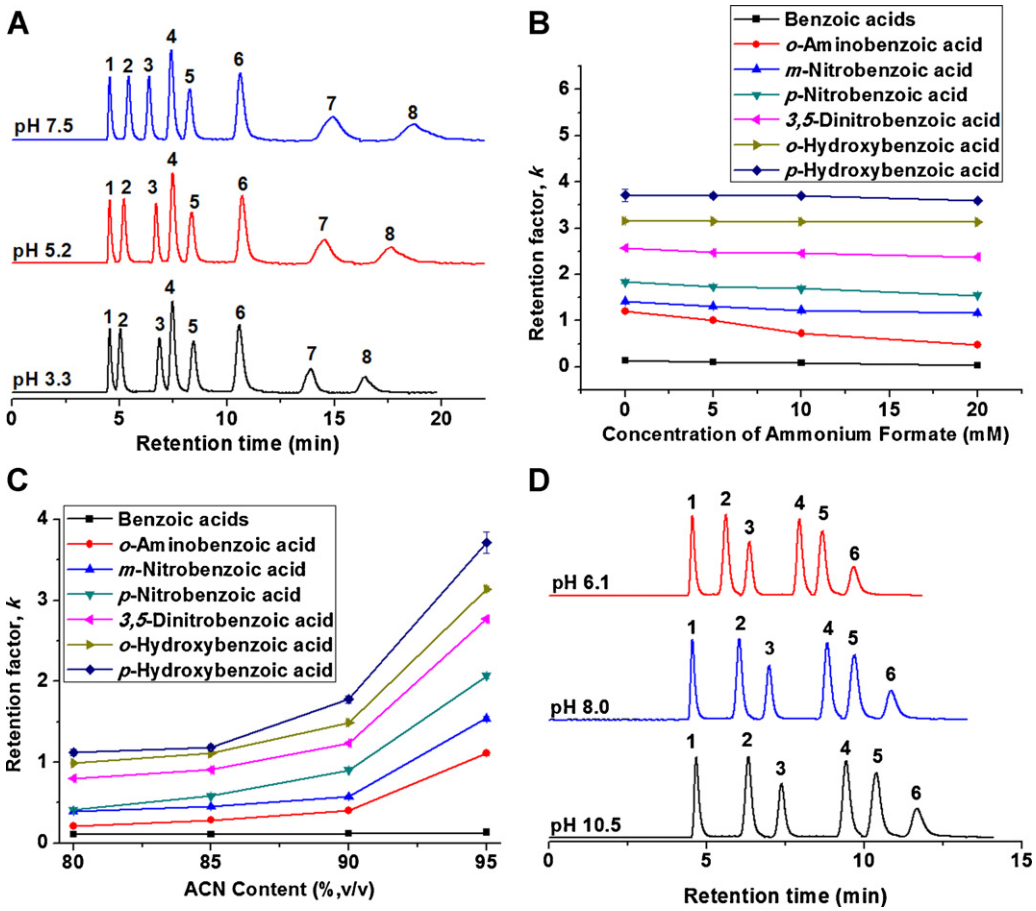



**Fig. 6.** Chromatograms of benzoic acids at different mobile phase pH (A), the retention factors of benzoic acids with different concentration of ammonium formate (B), ACN content (C), and (D) chromatograms of anilines at different mobile phase pH. Experimental conditions: 100 μm i.d. × 30 cm column; flow rate, 400 nL/min; injection volume, 20 nL; UV detection wavelength, 214 nm for benzoic acids, 254 nm for anilines. Mobile phase A: $H_2O$/ACN containing FA or ammonium hydroxide; peaks in A 1, toluene; 2, benzoic acids; 3, *o*-aminobenzoic acid; 4, *m*-nitrobenzoic acid; 5, *p*-nitrobenzoic acid; 6, 3,5-dinitrobenzoic acid; 7, *o*-hydroxybenzoic acid; 8, *p*-hydroxybenzoic acid; mobile phase B: ammonium formate (pH 7.5) containing 85% (v/v) ACN. Mobile phase C: $H_2O$/ACN (v/v), pH 7.0. Mobile phase D: $H_2O$/ACN containing FA or ammonium hydroxide, peaks in D: 1, toluene; 2, *o*-toluidine; 3, *N*,*N*-dimethylaniline; 4, *N*-methylaniline; 5, aniline; 6, *o*-nitroaniline.

affected by the change of pH (Fig. 6D). At low pH, the positively charged molecules of anilines had weak electrostatic repulsion with the dimethylamine of NAHAM, which led to weak retention. With the increased pH, the molar ratio of non-charged molecules to positively charged molecules increased, therefore, the electrostatic repulsion became weaker. At the mean time, with the increased percent of non-charged anilines, the strength of the hydrophilic interaction between analytes and stationary decreased. Generally, the migration time of anilines was longer, indicating that hydrophilic interaction between stationary phase and analytes was stronger slightly.

Moreover, the retention factors of anilines decreased with the increased ammonium formate concentration from 10 mM to 40 mM, which may be attributed to the increased hydrophilicity of stationary phase with an increase of salt concentration. This phenomenon was also reported in previous reports [47,48]. Furthermore, the retention of basic analytes increased with an increase of ACN content of the mobile phase, which clearly demonstrated that the monolithic column exhibited a hydrophilic chromatographic behavior towards basic analytes.

### 3.4. Reproducibility of monolithic column

The column efficiency was not apparently changed even after 100 times analysis. Both column-to-column and batch-to-batch reproducibilities for the prepared monolithic columns were also evaluated with the RSDs of retention times of the five analytes were 5.9 ($n = 5$) and 10.5% ($n = 3$), respectively. Moreover, the intra- and inter-batch RSDs for column efficiencies were 1.5% ($n = 4$) and 4.1% ($n = 3$), respectively. Besides, each monolith showed high stability and can be used repeatedly for at least 100 times without any decrease of the efficiency.

### 3.5. Application to analysis of nucleosides in urine

Nucleosides (cytidine, thymidine, adenosine, guanosine and uridine) are involved in the regulation and modulation of various physiological processes including in the central nervous system [49]. These nucleosides have been frequently analyzed by HPLC [49–51]. However, the determination of nucleosides is scarcely reported on *c*LC system. Therefore, in current study, we employed the advantages of *c*LC system and poly(NAHAM-*co*-PETA) monolith to the determination of nucleosides in urine. To improve the sensitivity of *c*LC, on-line preconcentration on *c*LC was used by the combination of an extraction monolith with an analytical monolithic column. In addition, automatic method allows flexible and precise manipulation of both extraction and separation processes [40,52].

According to our previous work, poly(AAPBA-*co*-EDMA) monolith can be used to effectively extract *cis*-*diol*-containing compounds under basic condition [35]. Due to the seperation of nucleosides on poly(NAHAM-*co*-PETA) monolith is

**Table 2**
Linearity characteristics of four nucleosides by *c*LC.

| Compounds | Linear range (μg/mL) | Regression line | | | LOD (μg/mL) | LOQ (μg/mL) |
|---|---|---|---|---|---|---|
| | | Slope | Intercept | $R^2$ | | |
| Uridine | 0.2–20 | 3270.5 | −3683.6 | 0.9947 | 0.044 | 0.15 |
| Adenosine | 0.2–20 | 3418.6 | −2134.9 | 0.9963 | 0.040 | 0.13 |
| Cytidine | 0.2–20 | 2742.4 | 485.3 | 0.9960 | 0.048 | 0.16 |
| Guanosine | 0.2–20 | 2526.5 | 50.8 | 0.9920 | 0.052 | 0.17 |

**Table 3**
Precision (intra- and inter-day) and recoveries for the determination of four nucleosides in urine samples.

| Compounds | Intra-day precision (RSD, %, $n = 5$) | | | Inter-day precision (RSD, %, $n = 3$) | | | Recovery (%, $n = 3$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Low (0.2 μg/ml) | Medium (5 μg/ml) | High (20 μg/ml) | Low (0.2 μg/ml) | Medium (5 μg/ml) | High (20 μg/ml) | Low (0.2 μg/ml) | Medium (5 μg/ml) | High (20 μg/ml) |
| Uridine | 6.9 | 7.3 | 7.5 | 9.4 | 7.3 | 8.6 | 106.8 ± 6.8 | 96.3 ± 5.6 | 99.2 ± 5.2 |
| Adenosine | 5.3 | 6.5 | 5.7 | 10.2 | 9.8 | 8.5 | 98.5 ± 5.9 | 92.3 ± 6.9 | 86.5 ± 3.6 |
| Cytidine | 7.4 | 5.1 | 8.3 | 9.9 | 8.7 | 9.4 | 94.6 ± 6.3 | 95.4 ± 7.8 | 89.7 ± 6.5 |
| Guanosine | 4.3 | 3.6 | 3.6 | 8.2 | 7.3 | 7.1 | 101.4 ± 7.5 | 89.9 ± 5.4 | 88.6 ± 4.7 |

**Table 4**
Quantification of four nucleosides in six urine samples.[a]

| Compounds | Healthy volunteers | | | | Tumor patients | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sample 1 | | Sample 2 | | Sample 3 | | Sample 4 | | Sample 5 | | Sample 6 | |
| | Detected (μg/mL) | Recovery % (0.4 μg/mL) | Detected (μg/mL) | Recovery % (0.4 μg/mL) | Detected (μg/mL) | Recovery % (0.4 μg/mL) | Detected (μg/mL) | Recovery % (0.4 μg/mL) | Detected (μg/mL) | Recovery % (0.4 μg/mL) | Detected (μg/mL) | Recovery % (0.4 μg/mL) |
| Uridine | n.q. | 88.6 | n.q. | 88.5 | 0.11 | 99.1 | 0.16 | 92.0 | 0.14 | 98.9 | 0.12 | 98.8 |
| Adenosine | n.q. | 89.7 | n.q. | 89.8 | 0.15 | 109.5 | 0.23 | 101.7 | 0.18 | 104.8 | 0.23 | 109.9 |
| Cytidine | n.d. | 99.1 | n.q. | 88.2 | n.q. | 98.0 | 0.13 | 97.2 | 0.20 | 87.6 | 0.15 | 99.1 |
| Guanosine | n.d. | 111.5 | n.d. | 92.1 | n.d. | 104.7 | n.q. | 106.5 | n.d. | 101.5 | n.q. | 109.1 |
| Total level | <0.20 | – | <0.20 | – | 0.26 | – | 0.52 | – | 0.52 | – | 0.50 | – |

[a] n.d., not detected; n.q., not quantified.

pH-independent, the combination of poly(AAPBA-*co*-EDMA) monolith and poly(NAHAM-*co*-PETA) monolith can further benefit the online SPME-cLC analysis of nucleosides.

### 3.5.1. Establishment of on-line extraction system

We assessed the on-line SPME-*c*LC system by optimizing several factors, including the loading flow rate, the eluent volume, the composition of the carrier solution and eluent.

Firstly, the loading flow rate can change the contact time of analytes on the poly(AAPBA-*co*-EDMA) monolith, which will generally influence the extraction efficiency. But in our case, when the loading flow rate increased from 0.01 to 0.1 mL/min, the extraction efficiency did not change much, however, the back pressure of poly(AAPBA-*co*-EDMA) monolith was dramatically increased from 2 to 11 MPa. When the flow rate increased over 0.1 mL/min, the back pressure elevated up to 15 MPa. Such high back pressure frequently caused the detachment of poly (AAPBA-*co*-EDMA) monolith. Therefore, the loading flow rate was maintained at 0.1 mL/min.

Secondly, the effect of the eluent volume on the sensitivity was investigated by changing the elution time from 10 to 30 s at constant flow rate 0.1 mL/min. It was showed that with the increase of elution time from 10 to 20 s, the signals of the four nucleosides were apparently increased. When the elution time was over 20 s, however, the signals of analytes changed, while the back pressure of separation module increased to over 18 MPa. Consequently, the elution time was fixed at 20 s.

Thirdly, we also investigated the composition of the carrier solution and the eluent. Based on our previous work [35], basic solution containing high ACN content (80%, v/v) was chosen as the carrier solution. Moreover, change of pH influenced little to the separation of nucleosides. Therefore, ACN/$H_2O$ (v/v, 80/20, adjust pH to 10.2 by adding ammonium hydroxide) and ACN/$H_2O$ (v/v, 98/2, adjust pH to 3.5 by adding FA) were chosen as carrier solution and eluent, respectively.

The loading sample volume was also assessed from 0.05 to 1 mL. The results showed that with the increase of loading sample volume, the signal of nucleosides kept increasing, while no obvious signal increase was observed when the loading sample volume was over 0.8 mL. Thus, the loading sample volume was maintained at 0.8 mL.

Fig. 7A showed the chromatogram of four target analytes by on-line SPME-*c*LC under the optimized conditions or by direct injection on *c*LC system. Noticeably, the poly(AAPBA-*co*-EDMA) monolith only had strong interaction with *cis*-diol-containing compounds (uridine, adenosine, cytidine, guanosine). Thus, these four analytes were selected for further methodological study.

### 3.5.2. Methodological investigation

The limits of detection (LODs) and the limits of quantification (LOQs) were calculated as the concentration of the analytes at a signal-to-noise ratio (S/N) of 3 and 10, respectively. As shown in Table 2, the LODs and LOQs for the four nucleosides were in the range of 0.04–0.052 μg/mL and 0.13–0.17 μg/mL, respectively. To evaluate the linearity of the measurement, the analytes with various concentrations ranging from 0.2 to 20 μg/mL were analyzed. As shown in Table 2, good linearities were obtained for the determination of all compounds, with linear coefficient $R^2$ values of >0.99. In addition, the reproducibility of the developed method was evaluated by investigating the intra and inter-day precisions. Results showed that the intra- and inter-day relative standard deviations (RSDs) are less than 8.3 and 10.2%, respectively (Table 3). The recoveries were measured by analyzing the spiked urine samples at three different concentrations ranging from 0.2 to 20 μg/mL. Our

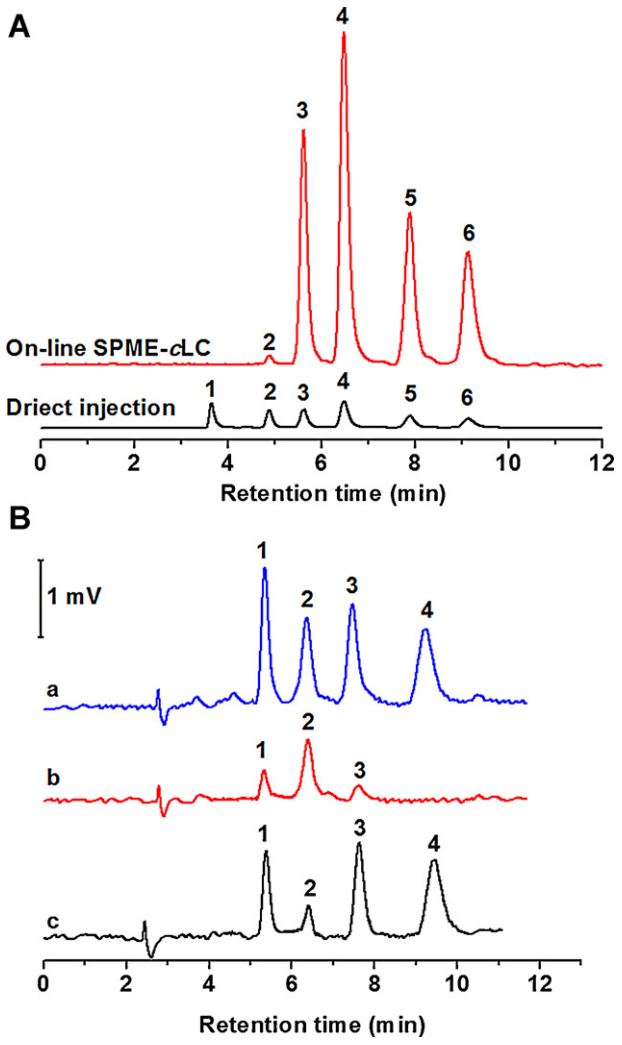



**Fig. 7.** Chromatograms of nucleosides (A) with on-line SPME-*c*LC (a) or direct injection (b). (B) Chromatograms of nucleosides spiked urine sample (a), urine sample (b), nucleosides standard (c). Peaks in A: 1, toluene; 2, thymidine; 3, uridine; 4, adenosine; 5, cytidine; 6, guanosine. Peaks in B: 1, uridine; 2, adenosine; 3, cytidine; 4, guanosine; experiment conditions: 100 μm i.d. × 30 cm column; UV detection wavelength, 254 nm; flow rate, 400 nL/min; direct injection volume, 20 nL; on-line SPME injection, 0.8 mL; mobile phase: ACN/$H_2O$ (95/5, v/v) containing FA, pH 3.5.

results showed that the recoveries were in the range of 86.5–106.8% (Table 3).

### 3.5.3. *Application to urine analysis*

Fig. 7B shows the typical chromatograms of nucleosides obtained by on-line SPME-*c*LC analysis of urines from tumor patients. The four nucleosides in urine samples were determined by comparing the retention time of the analytes to that of the standards and standards spiked urine samples. The quantitative results of urine samples from 4 tumor patients and 2 health volunteers were summarized in Table 4 with the recoveries between 87.6 and 111.5%. As shown in Table 4, the total levels of nucleosides (>0.2 μg/mL) in urines from tumor patients were relatively higher than that of healthy volunteers.

## 4. Conclusion

A poly(NAHAM-*co*-PETA) monolithic column was successfully prepared for *c*HILIC. The characteristics and its performance for the separation of neutral polar and charged compounds were evaluated in detail. Efficient separations of five nucleosides, seven benzoic acids, and five anilines were achieved by using the poly(NAHAM-*co*-PETA) monolith. In addition, by combining the poly(NAHAM-*co*-PETA) monolith with poly(AAPBA-*co*-EDMA) extraction monolith, an on-line SPME-*c*LC system was successfully applied in rapid and sensitive determination four nucleosides from urine samples. This method developed in current study offers a potential application for clinical diagnose. Studies are currently under investigation to couple with MS and electrochemical detection.

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