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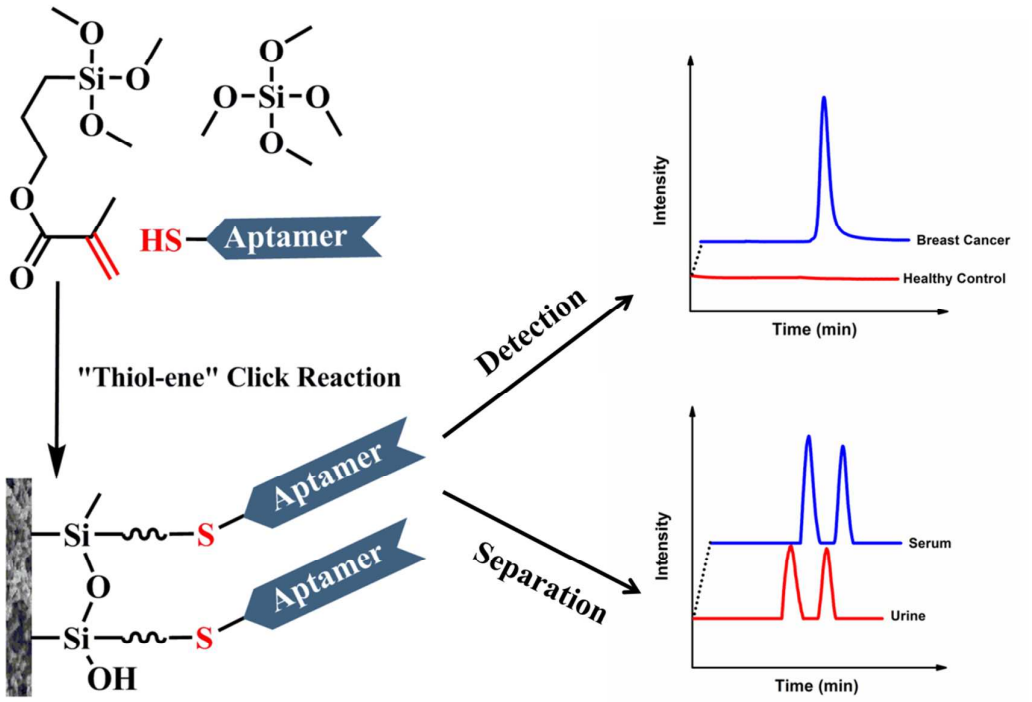

Preparation of aptamer-based organic-silica hybrid monolithic capillary column by "thiol-ene" click chemistry for detection of enantiomers of chemotherapeutic anthracyclines.

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Facile One-pot Synthesis of Aptamer-Based Organic-Silica Hybrid Monolithic Capillary Column by "Thiol-ene" Click Chemistry for Detection of Enantiomers of Chemotherapeutic Anthracyclines

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ABSTRACT: In the current study, we developed a facile strategy for one-pot synthesis of aptamer-based organic-silica hybrid monolithic capillary column. A 5'-SH-modified aptamer specifically targeting doxorubicin was covalently modified in the hybrid silica monolithic column by sol-gel method combined with "thiol-ene" click reaction. The prepared monolithic column had good stability and permeability, large specific surface, and showed excellent selectivity towards chemotherapeutic anthracyclines of doxorubicin and epirubicin. In addition, the enantiomers of doxorubicin and epirubicin can be well separated by aptamer-based affinity monolithic capillary liquid chromatography. Furthermore, doxorubicin and epirubicin spiked in serum and urine also were successfully determined, which suggested that the complex biological matrix had neglect effect on the detection of doxorubicin and epirubicin. Finally, we quantified the concentration of epirubicin in the serum of breast cancer patients treated with epirubicin by intravenous injection. The developed analytical method is cost-effective and rapid, and the biological samples can be directly analyzed without any tedious sample pretreatment, which is extremely useful in monitoring the medicines in serum and urine for the pharmacokinetic studies.

1. Introduction

Aptamers are synthetic nucleic acids that normally can form three-dimensional conformation and have the capability to specifically recognize and bind to target molecular.[1, 2] Since the first discovery in 1990s, aptamers have been widely used in various fields, including biosensing,[3-5] clinical disease diagnosis[6, 7] and treatment,[8, 9] protein purification[10, 11] and medicine development.[12, 13]

Affinity chromatography is a powerful technique in separation, preconcentration and detection of compounds from complex mixture.[14-16] And antibody-antigen interaction is frequently used in affinity chromatography.[17] Whereas, the immobilized antibodies are prone to degradation and denaturation; and the large molecular size of antibodies limits the surface loading and therefore column capacity. Compared to antibodies, aptamers exhibit significant advantages of good stability, easy synthesis, and facile modification and immobilization,[18, 19] which makes aptamers promising alternative to antibodies in affinity chromatography.

Several methods have been developed to prepare aptamer modified columns. Aptamer can be immobilized on the inner surface of open-tubular capillary column,[20, 21] or on the particles in packed column.[22] The immobilization capacity of aptamer is limited in open-tubular capillary column, and the packing of particles in packed column is labour intensive. Monolithic columns, which display fast mass transfer rate, larger loading capacity than open-tubular capillary column and easier preparation than packed columns, could be a good choice to prepare aptamer modified columns.

Up to date, several strategies have been developed to prepare aptamer modified monolithic capillary columns. Zhao et al.[23, 24] immobilized biotinylated DNA aptamer to the streptavidin-modified polymer monolithic capillary column. Deng et al.[25] and Han et al.[26] prepared aptamer modified monolithic capillary columns by covalently bonding 5'-NH_2-modified aptamer to the column via glutaraldehyde for the detection of human α-thrombin or lysozyme. Whereas, these preparation methods involved in multiple steps and required pre-modification of the monolithic capillary column for the subsequent immobilization of aptamer, which is therefore relatively labour intensive and time-consuming.

Recently, our group successfully developed a method to prepare organic-silica hybrid monolithic capillary column by sol-gel combined with "thiol-ene" click reaction, which allows higher yield and less by-products and can be performed under the mild conditions.[27] In this respect, the "thiol-ene" click reaction could be a promising strategy for the preparation of aptamer modified organic-silica hybrid monolithic capillary column with one-pot synthesis.

Herein, we prepared apatmer modified monolithic capillary column using tetramethoxysilane (TMOS) as the silane coupling reagent and γ-methacryloxypropyltrimethoxylsilane (γ-MAPS) as the functional silane coupling reagent, which can readily react with a 5'-SH-modified DNA aptamer via "thiol-ene" click reaction (Figure 1). As a proof-of-concept, we used the aptamer that was previously selected to specifically target doxorubicin[28] (Figure 2), which is widely used in cancer chemotherapy. Using the sol-gel combined with "thiol-ene" click reaction, the aptamer modified monolithic capillary column can be easily prepared in one-pot. And the column

showed excellent selectivity towards doxorubicin and its enantiomer of epirubicin. In addition, the enantiomers of doxorubicin and epirubicin can be well separated by the aptamer modified monolithic column. We then further successfully determined the epirubicin concentration in the serum of breast cancer patients treated with epirubicin. Due to the high selectivity of the aptamer to target compounds, biological samples can be directly analyzed without any tedious sample pretreatment, which is therefore extremely useful in monitoring the medicines in serum and urine for the pharmacokinetic studies.

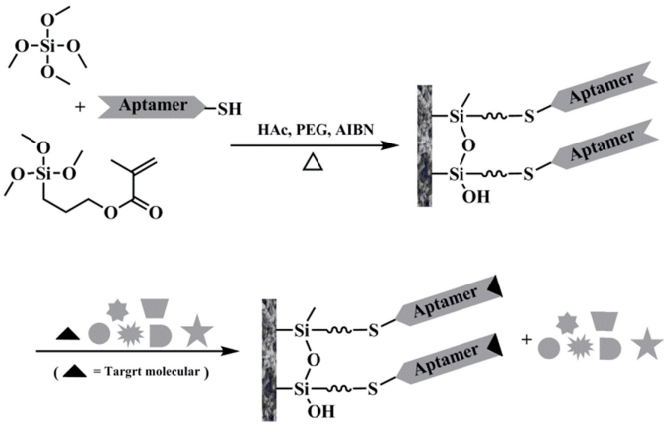

Figure 1. Schematic procedure for the preparation of aptamer-based organic-silica hybrid monolithic capillary column by sol-gel method combined with "thiol-ene" click reaction.

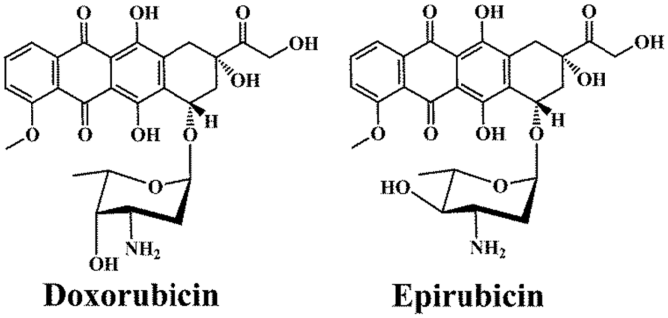

Figure 2. The structures of the enantiomers of doxorubicin and epirubicin.

2. Experimental

2.1 Chemicals and Materials

Fused-silica capillary (75 μm i.d. × 360 μm o.d.) was purchased from Yongnian Optic Fiber Plant (Hebei, China). Tetramethoxysilane (TMOS) and γ-methacryloxypropyltrimethoxylsilane (γ-MAPS) were purchased from Wuhan University Silicone New Material (Wuhan, China). Azobisisobutyronitrile (AIBN) and poly(ethylene glycol) with the molecular weight of 6000 (PEG-6000) were purchased from Shanghai Chemical Reagent Corporation (Shanghai, China). AIBN was purified by recrystallization from ethanol at 40 °C. Organic solvents were all of HPLC grade. The water used throughout all experiments was purified using a Milli-Q apparatus (Millipore, Bradford, USA). Doxorubicin and epirubicinwere purchased from Aladdin Industrial Corporation (Shanghai, China). The single-stranded DNA aptamer targeting doxorubicin and epirubicin (5'-ACCATCTGTGTAAGGGGTAAGGGGTGGGGGTGGGTACGTC T-3', denoted as Aptamer-41) and control oligonucleotide (5'-ACAAGCCGTCTATTCGATTACCGTTGAGCCTGATTATGTGT -3', denoted as control ssDNA), with 5'-end modified by SH through a C6-carbon spacer, were synthesized by Takara Biotechnology (Dalian, China). All other reagents were obtained from various commercial sources and were of analytical grade unless otherwise indicated.

2.2 Preparation of Aptamer-based Organic-silica Hybrid Monolithic Capillary Columns

To activate the silanol groups, the fused-silica capillaries were sequentially washed with 1 M NaOH for 2h, H_2O for 30 min, 1 M HCl for 1 h, H_2O for 30 min and methanol for 30 min followed by drying under nitrogen flow at 160 °C for 6 h.

A polymerization mixture containing acetic acid (HAc) (0.01 M, 500 mg), PEG-6000 (45 mg), TMOS (185 mg), γ-MAPS (15 mg), Aptamer-41 (15 μL, 0.26 nmol/μL) and AIBN (1 mg) was completely mixed and degassed by a 5-min ultra-sonication. The mixture was then manually introduced into the fused-silica capillary (10 cm-long, 75 μm i.d. × 360 μm o.d.) by a syringe. After both ends of the capillary were sealed with two pieces of silicone rubber, the mixture was incubated at 40 °C for 12 h for simultaneous polymerization and "thiol-ene" click reaction. The resulting monolith was completely flushed with water and ACN sequentially to remove the PEG-6000 and other residuals.

For comparison, an organic-silica hybrid monolith without aptamer modification and an organic-silica hybrid monolith modified with the control ssDNA were also prepared. The preparation conditions were the same as that for the Aptamer-41 modified organic-silica hybrid monolith.

2.3 Characterization of Aptamer-based Organic-silica Hybrid Monolithic Capillary Column

The specific surface area of the prepared aptamer-based organic-silica hybrid monolithic materials were measured by nitrogen adsorption-desorption experiments using a JW-BK specific 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 to 120 °C for 4 h to remove the physically adsorbed substances. Specific surface area values were determined by the Brunauer-Emmett-Teller (BET) equation at P/P_0 between 0.05 and 0.3.[29]

The microscopic morphology of the monoliths was examined by scanning electron microscopy (SEM) using a Quanta 200 scanning electron microscope (FEI Company, Holland).

Permeability of the monolith was measured using a Shimadzu LC-20AB pump (Kyoto, Japan). ACN was pumped through the prepared monolithic columns (10 cm-long, 75 μm i.d.× 360 μm o.d.) at the flow rate of 10 μL/min. The back-pressure was recorded when the pressure stabilized. The permeability (K) was calculated according to Darcy's Law[30] by the following equation,

$$K = u\eta L/\Delta P,$$

where u (m/s) is the linear velocity of mobile phase, η is the viscosity of mobile phase (it is 0.38×10^{-3} Pa s for ACN at 20 °C in this work), L (m) is the length of the monolithic column and ΔP (Pa) is the pressure drop across the monolithic column.

2.4 Aptamer-Based Hybrid Affinity Monolithic Capillary Liquid Chromatography

All the aptamer-based hybrid affinity monolithic capillary liquid chromatography experiments were performed on a HPLC system consisting of a Shimadzu LC-20AB binary pump (Tokyo, Japan), a Shimadzu injection valve equipped with a 20 μL sample loop (Tokyo, Japan), one GL Sciences MU 701 UV-vis detector with a 6 nL detection cell (Tokyo, Japan). To achieve micro-flow rate of 1

μL/min for separation, a T-union with one end connected to the monolithic column and the other end connected to a capillary (8 cm-long, 75 μm i.d.) was employed as a flow splitter between the pump and injection valve. All the experiments were performed at 20 °C. All the chromatograms were recorded at a wavelength of 254 nm. Mobile phase A consisted of 20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (pH 7.4). Mobile phase B was 2 M $NaClO_4$ solution. The mobile phase gradient was 0-5 min, 0% B, 5-10 min, 100% B.

2.5 Chiral Separation of Doxorubicin and Epirubicin by Aptamer-based Organic-silica Hybrid Monolithic Capillary Column

The separation experiment was performed on the same apparatus used for the aptamer-based hybrid affinity monolithic capillary liquid chromatography. Mobile phase A consisted of 20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (pH 7.4). Mobile phase B was 1 M $NaClO_4$ solution. The chiral separation of doxorubicin and epirubicin was performed using the gradient elution of 0-5 min, 0% B, 5-20 min, 0%-100% B.

2.6 Evaluation of the Aptamer Coverage Density and Stability of Aptamer-based Organic-silica Hybrid Monolithic Capillary Column

The aptamer coverage density was evaluated by breakthrough curve according to previous reported method.[31] Briefly, the aptamer-modified monolith (10 cm-long) was equilibrated with the mobile phase A (20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, pH 7.4); and then mobile phase A containing doxorubicin (10 pmol/μL) was pumped into the column at a flow rate of 1 μL/min. Every 2 μL of effluent liquid was collected and measured by a Nano UV-Vis spectrophotometer (METASH B500, Shanghai, China). The breakthrough curve was constructed by plotting the intensity of UV absorbance versus the effluent volume.

The aptamer-based organic-silica hybrid monolithic capillary column was used three times per week for four weeks to evaluate the stability under the same conditions. After each analysis, the column was rinsed with the mobile phase A (20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, pH 7.4) for 30 min and then stored at 4°C.

2.7 Determination of Epirubicin in the Serum of Breast Cancer Patients

The study included 10 breast cancer female patients and 5 healthy female controls which were all collected from Zhongnan Hospital of Wuhan University, China. Patients with histologically confirmed invasive carcinoma of the breast with stage I to III received epirubicin treatment at a dose of 100~185 mg/m^2 on the 1st day of the 1st cycle of chemotherapy. Blood samples were collected within 10 days after the administration. Controls were the participants in a routine health check-up program in the Examination Center, Zhongnan Hospital of Wuhan University, China. All blood samples were collected with adding heparin after 8-14 hours of an overnight fast. Urine samples were collected from the healthy persons. Written informed consent was obtained from the study subjects, and an approval was granted by the Zhongnan Hospital Ethics Committee and met the declaration of Helsinki.

All serum and urine samples were filtered to remove the undissolved substance by PRECLEANTM Syringe Filter Nylon membrane (13 mm × 0.22 μM, ANPEL Scientific Instrument Co., Shanghai) prior to analysis.

3. Results and discussion

3.1 Characterization of Aptamer-based Organic-silica Hybrid Monolithic Capillary Column

The morphology of aptamer-based organic-silica hybrid monolith was examined by scanning electron microscopy (SEM). As shown in Figure 3A, the formed monolith was homogeneous and attached well to the inner wall of the capillary, which can provide effective mass transfer and high stability of column. The specific surface area of the aptamer-based organic-silica hybrid monolith was 257 m^2/g with a 4.5 nm mesoporous distribution (Figure 3B).

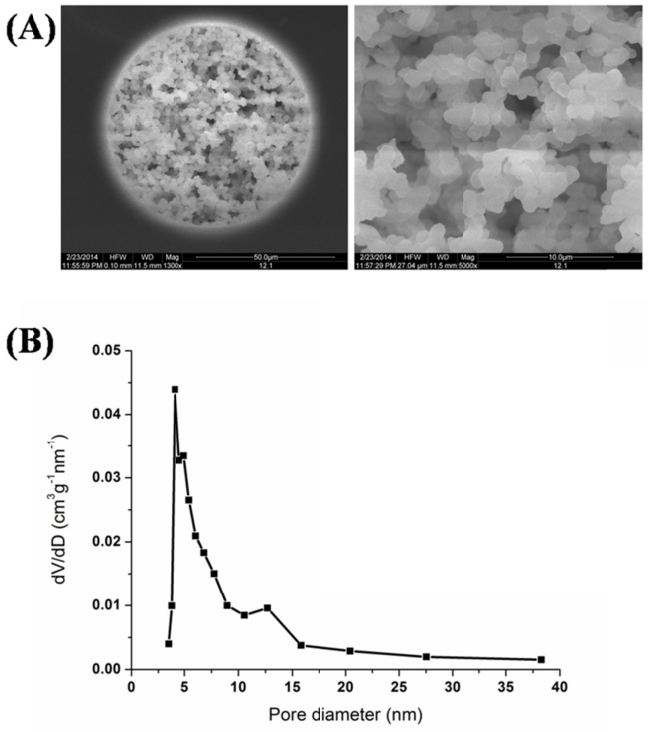

Figure 3. (A) Scanning electron microscope images of aptamer-based organic-silica hybrid monolithic capillary column. Left: 1300×wide-view. Right: 5000×close-up-view. (B) Mesoporousdistribution of the aptamer-based monolithic column.

3.2 Specificity of Aptamer-based Organic-silica Hybrid Monolithic Capillary Column to Doxorubicin and Epirubicin

Doxorubicin and epirubicin were used to evaluate the specificity of the aptamer modified monolithic column. The results showed both doxorubicin and epirubicin retained well on the aptamer modified monolith; after switching the mobile phase to 2 M $NaClO_4$, which can destroy the three-dimensional conformation of the aptamer, doxorubicin and epirubicincan be fast eluted out (Figure 4A). Whereas, doxorubicin and epirubicin had no retention on the monolithic column without modification (Figure 4B) or modified by control ssDNA (Figure 4C). The results suggested that the retention of doxorubicin and epirubicin is mainly due to the aptamer modified in the monolithic column.

Furthermore, we chose other 12 analytes, including commonly used drugs (amoxicillin, penicillin, chloramphenicol), environmental pollutants (sulfapyridine, sulfadoxine, sulfameter, benzopyrene, benzofluoranthrene, benzanthracene, trimethoprim), and endogenous compounds (2'-deoxyadenosine, NAD) that may exist in serum and urine, together with doxorubicin and epirubicinto further evaluate the selectivity of the aptamer modified column. As shown in Figure 5, except for doxorubicin and epirubicin, all the other compounds

had no retention on the aptamer modified column, which suggested the high selectivity of the aptamer modified column towards doxorubicin and epirubicin.

We also optimized the amounts of Aptamer-41 in the preparation of aptamer modified monolithic column. The results showed that the maximum banding capacity can be achieved when 3.9 nmol of Aptamer-41 was used (Figure S1, Supporting Information).

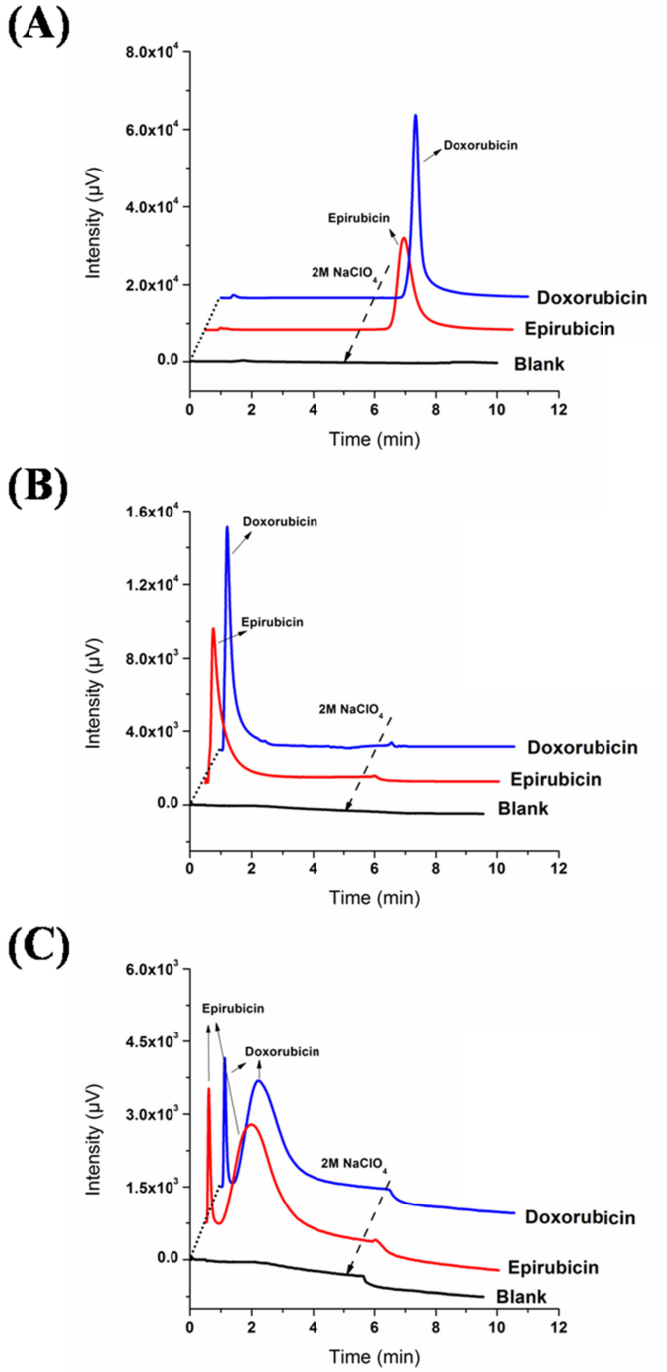

Figure 4. Chromatograms of doxorubicin and epirubicinon in (A) aptamer-based organic-silica hybrid monolithic capillary column, or (B) organic-silica hybrid monolithic capillary column without aptamer modification, or (C) aptamer-based organic-silica hybrid monolithic capillary column modified with the control ssDNA. Monolithic capillary column: 10 cm-long, 75 μm i.d. × 360 μm o.d.; flow rate, 0.5 mL/min; inject volume, 20 μL; sample concentration, doxorubicin (200 μg/mL), epirubicin (100 μg/mL); splitting ratio, 1/500; detection wavelength, 254 nm.

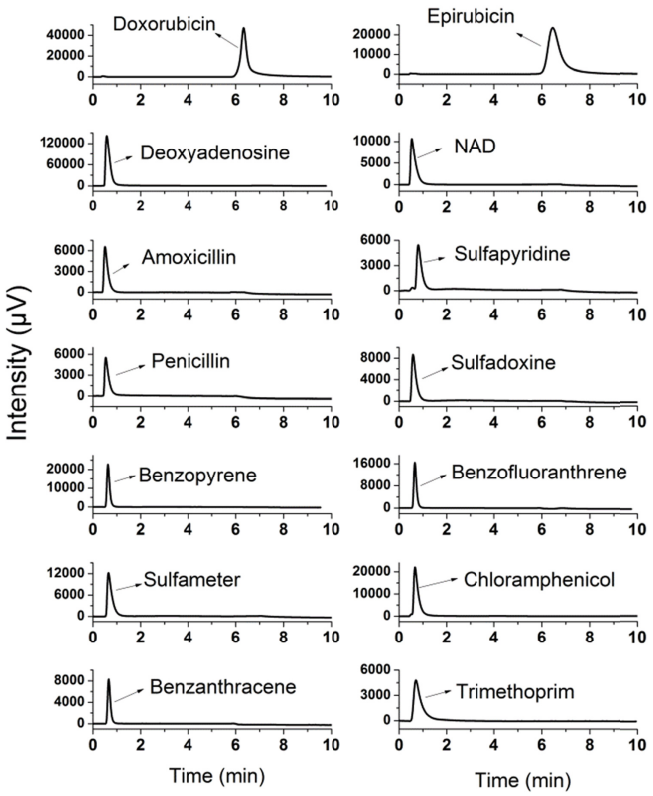

Figure 5. Evaluation of the specificity of the aptamer-based organic-silica hybrid monolithic capillary column. Monolithic capillary column: 10 cm-long, 75 μm i.d. × 360 μm o.d.; flow rate, 0.5 mL/min; inject volume, 20 μL; splitting ratio, 1/500; detection wavelength, 254 nm.

3.3 Method Validation

The calibration curves were constructed by plotting the mean peak area of target analytes spiked in serum or urine from healthy controls versus the concentrations based on data obtained from triplicate measurements. The results showed that good linearities within the range of 5 - 1000 μg/mL of target analytes were obtained with a correlation coefficient values (R) being great than 0.99 (Tables S1, Supporting Information). The on column limit of detection (LOD) and the limit of quantification (LOQ), defined as the amounts of the analytes at a signal-to-noise ratio (S/N) of 3 and 10, respectively, were 10 pg and 34 pg for both doxorubicin and epirubicin (Table S1, Supporting Information).

Shown in Figure 6 are the typical chromatograms of doxorubicin and epirubicin spiked in either serum or urine. The results showed that doxorubicin and epirubicin can be distinctly detected from serum and urine by nanoLC-UV. And the complex matrices of serum or urine had no effect on the determination of doxorubicin and epirubicin, which could be attributed to the high selectivity of the aptamer modified in the monolithic capillary column.

The recoveries of doxorubicin and epirubicin spiked in serum and urine samples were determined to be in the range of 90.1 to 97.8% (Table S2, Supporting Information), which can satisfy the

measurements. The reproducibility of the method was evaluated by the measurement of intra- and inter-day precisions. The intra- and inter-day relative standard deviations (RSDs) were calculated by measuring three different concentrations of target analytes at 25 μg/mL, 100 μg/mL and 500 μg/mL. Three parallel analysis over a day gave the intra-day RSDs, and the inter-day RSDs were determined by 3 consecutive days. The results showed that the intra- and inter-day RSDs were less than 3.7% and 2.6%, respectively (Table S3, Supporting Information), demonstrating that good reproducibility was achieved.

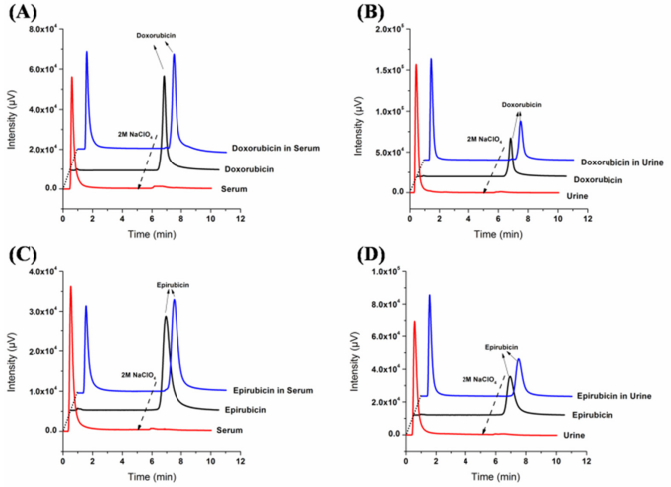

Figure 6. Chromatograms of doxorubicin and epirubicin spiked in serum and urine from healthy controls. (A) Analysis of doxorubicin spiked in serum. (B) Analysis of doxorubicin spiked in urine.(C) Analysis of epirubicin spiked in serum. (D) Analysis of epirubicin spiked in urine. Monolithic capillary column: 10 cm-long, 75 μm i.d. × 360 μm o.d.; flow rate, 0.5 mL/min; inject volume, 20 μL; sample concentration, doxorubicin (200 μg/mL), epirubicin (100 μg/mL); splitting ratio, 1/500; detection wavelength, 254 nm.

3.4 Chiral Separation of Doxorubicin and Epirubicin

Separation of enantiomers is still a challenge in separation sciences.[32] Doxorubicin and epirubicin are enantiomers and the only difference between them is that the conformation of 4'-OH on the aminoglycoside of epirubicin is anti-form while the doxorubicin is cis-from, which may offer different affinity between analytes and aptamer. Here we tried to separate doxorubicin and epirubicin using the aptamer modified monolith. In this respect, we mixed doxorubicin and epirubicin in water, serum or urine samples. We optimized the separation conditions by changing the concentration of the $NaClO_4$ (mobile phase B) and the elution gradient. The results showed that doxorubicin and epirubicin can be well separated in water (Figure 7A), serum and urine samples (Figure 7B) under optimized separation conditions, which was, to the best of our knowledge, the first report for the successful aptamer-based separation of doxorubicin and epirubicin.

In the past few decades there is an increasing demand for the separation of chiral compounds in the pharmaceutical industry. Hitherto, a number of strategies have been developed and applied for the separation of the enantiomeric composition of chiral compounds.[33-35] In the current work, we demonstrated that the aptamer modified organic-silica hybrid monolithic capillary column can be successfully used to discriminate and separate enantiomers, which offers a new strategy in the separation of chiral compounds.

3.5 Aptamer Coverage Density and Stability of Aptamer-based Organic-silica Hybrid Monolithic Capillary Column

The breakthrough curve suggested that the saturated binding capability of the aptamer-modified monolithic column was 80.9 pmol of doxorubicin (Figure S2, Supporting Information). By assuming that each aptamer can bind one analyte, we calculated that the aptamer coverage density in the monolithic column was 40.5 pmol/mg. Since the initial aptamer density was 52 pmol/mg, the effective utilization percentage of aptamer was therefore 78%.

The aptamer-based organic-silica hybrid monolithic capillary column was used three times per week for four weeks to examine the stability, which was evaluated by the RSDs of retention time and peak height of the doxorubicin and epirubicin. The results showed that the RSDs of the retention time of doxorubicin and epirubicinwere 2.5% and 2.7% and the RSDs of peak heights of doxorubicin and epirubicin were 5.3% and 5.6%, demonstrating that the aptamer modified monolith maintained good stability (Figure S3, Supporting Information).

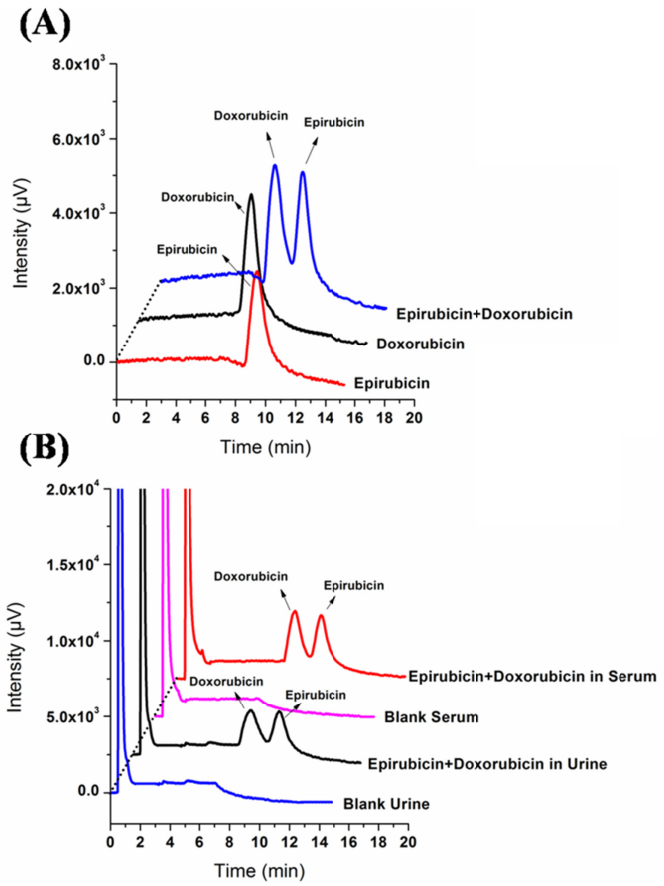

Figure 7. Separation of the enantiomers of doxorubicin and epirubicin spiked in water (A) or spiked in serum and urine (B). Monolithic capillary column: 10 cm-long, 75 μm i.d. × 360 μm o.d.; flow rate, 0.5 mL/min; inject volume, 20 μL; splitting ratio, 1/500; detection wavelength, 254 nm.

3.6 Determination of Epirubicin in the Serum of Breast Cancer Patients by Aptamer-Based Hybrid Affinity Monolithic Capillary Liquid Chromatography

We further applied the aptamer modified monolithic column to determine epirubicin in the serum of breast cancer patients treated with epirubicin by intravenous injection. The results showed that epirubicin can be distinctly detected and quantified in all the breast cancer patients; whereas, no epirubicin was found in the control

samples (Table 1). Shown in Figure 8 are the typical chromatograms for the detection of epirubicin from breast cancer patient and healthy control. The analytical method is cost-effective and the whole analytical procedure takes only 10 min, which therefore can be potentially used in monitoring anthracycline drug of epirubicin for the pharmacokinetic studies.

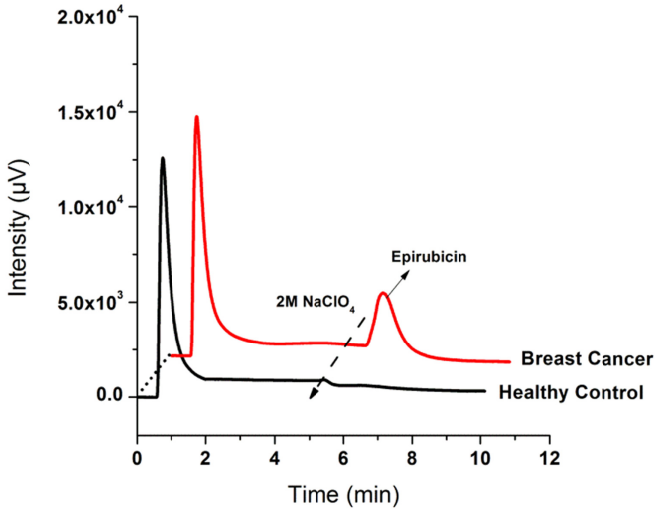

Figure 8. Chromatograms for the determination of epirubicin in the serum of breast cancer patients treated with epirubicin by intravenous injection.

Table 1. Measured epirubicin concentrations in the serum of healthy controls or breast cancer patients treated with epirubicin by intravenous injection.

Samples	Concentration (μg/mL)	RSD (%)
B1	58.2±1.3	1.7
B2	85.4±2.9	2.9
B3	33.0±2.3	4.6
B4	41.1±0.2	0.3
B5	39.2±2.2	3.9
B6	16.4±0.2	0.6
B7	23.6±2.2	5.7
B8	16.6±0.4	1.1
B9	18.2±0.3	0.8
B10	9.8±0.9	3.3
N1	n.d.	-
N2	n.d.	-
N3	n.d.	-
N4	n.d.	-
N5	n.d.	-

B1 to B10, the samples number of breast cancer patients; N1 to N5, the samples number of healthy controls; n.d., not detected.

4. Conclusions

We developed a facile strategy for one-pot synthesis of aptamer-based organic-silica hybrid monolithic capillary column, which had good stability and permeability, large specific surface, and showed excellent selectivity towards chemotherapeutic anthracyclines of doxorubicin and epirubicin. In addition, the enantiomers of doxorubicin and epirubicin can be well separated by aptamer-based hybrid affinity monolithic capillary liquid chromatography. Furthermore, doxorubicin and epirubicin spiked in serum and urine also were successfully determined, which suggested that the complex biological matrix had neglect effect on the detection of doxorubicin and epirubicin. Finally, we quantified the concentration of epirubicin in the serum of breast cancer patients treated with epirubicin by intravenous injection. The developed analytical method is cost-effective and rapid, which therefore can be potentially used in monitoring medicines for the pharmacokinetic studies.

Acknowledgements

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Notes and references

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