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A novel click lysine zwitterionic stationary phase for hydrophilic interaction liquid chromatography

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ABSTRACT

A novel type of zwitterionic HILIC stationary phase was prepared by covalently bonding the L-azido lysine on silica gel *via* click chemistry. The key intermediate azido lysine was synthesized by transformation the amino group in L-Boc-lysine to corresponding azido group and subsequent removal of the N-protected group (Boc). Finally, the azido lysine was covalently bonded to silica beads by click chemistry to get click lysine. Its structure was confirmed by FT-IR and elemental analysis. The new stationary phase showed good HILIC characteristics, when it was applied to separate polar and hydrophilic compounds, such as organic acids, cephalosporins and carbapenems. Compared with the commercial stationary phases, such as Atlantics HILIC and ZIC-HILIC, click lysine displayed better or similar chromatographic behaviors.

1. Introduction

Reverse phase liquid chromatography (RPLC) stationary phases (C18 and C8) are usually used in analysing variety of samples, including some highly polar and hydrophilic compounds, such as cephalosporins and carbapenems [1–8]. However, in these cases, highly water concentrated mobile phases (around 94%) are usually required to achieve better retention [9], which may result the lengthened equilibration of variable retention with multiple runs.

Since HILIC was proposed by Alpert in 1990 [10], its application in the analysis and separation of highly polar and hydrophilic compounds has been dramatically increased. Highly polar zwitterionic HILIC stationary phases possess polar groups bearing both positive and negative charges and they are capable of solvating polar and charged compounds (such as β-lactam antibiotics) *via* weak electrostatic interactions [11], which provides the chromatographer with a larger degree of freedom for choosing among buffer salts and ionic strength in method development. Furthermore, zwitterionic HILIC can provide a selectivity benefiting from both hydrophilic and weak electrostatic interactions, while maintaining a low eluent ionic strength to make the column an ideal choice for LC/MS analysis [12–16]. In addition, volatile organic mobile phase used in zwitterionic HILIC can increase the sensitivity with LC/MS applications [17,18]. Recently, some polymer and silica based zwitterionic stationary phases have been prepared and applied in the separation of oligosaccharides, peptides and basic compounds, as well as glycopeptide enrichment [19–28]. All of these stationary phases showed HILIC chromatography behaviors, but no single sub-technique can separate all kinds of solutes due to the diversity and complexity of the samples. Furthermore, most of the zwitterionic HILIC stationary phases are zwitterionic phosphorylcholine and betaine type. Up to now, only a few of α,β-amino acid type of zwitterionic stationary phases have been reported [29]. On the other hand, Cu(I) catalyzed Huisgen azide-alkyne 1,3-dipolar cycloaddition (CuAAC) reaction showed great power in variant fields, such as material sciences, organic synthesis and drug discovery, considerable applications have been reviewed [30–45]. So far, several covalently bonded stationary phases have been prepared by CuAAC, and they are utilized for analyzing complex samples under two dimensional liquid chromatography [46], separation highly polar compounds under HILIC model and enrichment of glycopeptides [19,34,35,47–51]. Herein, we would like to describe a zwitterionic amino acid stationary phase prepared by CuAAC. The chromatographic evaluation showed that this novel stationary phases exhibited good HILIC characteristics, and its application in the separation of hydrophilic cephalosporins and carbapenems under HILIC model is expected.

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2. Experiment

2.1. Chemicals and reagents

Spherical silica (5 μm particle size; 10 nm pore size; 300 $m^2 g^{-1}$ surface area, Fuji Silysia Chemical Ltd., Kasugai, Japan). HPLC grade acetonitrile (Sigma–Aldrich, St. Louis, MO, USA), formic acid (Acros Organics, New Jersey, USA) and methanol (Tedia, Fairfield, CT, USA). Milli-Q (Millipore, Bedford, MA, USA) ultrapure water was used throughout the HPLC experiment. L-Boc-lysine, sulfonyl chloride, sodium azide, acetylchloride, TFA, $CuSO_4 \cdot 5H_2O$, ammonium formate and sodium ascorbate were all domestic reagent (Ninfeng Chemical Reagent Ltd. and National Medicine Corporation Chemical Reagent Ltd., Shanghai, China) and used without further purification. 3-Isocyanatopropyl-triethoxysilane and propargylamine (National Medicine Corporation Chemical Reagent Ltd., Shanghai, China) were purified by distillation before use. The solvent of N-dimethyl formamide, acetonitrile, methanol and ethanol (Ninfeng Chemical Reagent Ltd., Shanghai, China) was distilled from calcium hydride before use. The test solutes used for HILIC evaluation were commercially available (test compounds such as uracil, adenosine, uridine, cytosine, cytidine, guanosine, cytosine, adenosine, guanosine, salicylic acid, orotic acid, procainamide, *p*-chlorosalicylic acid, *p*-aminobenzoic acid, sorbic acid, anisic acid, cinnamic acid, benzoic acid are from Aladdin Reagent Ltd., Shanghai, China, and ceftazidime pentahydrate, cefotaxime sodium, cefradine, cefepime hydrochloride, cefazolin sodium, cefprozil, cefpiramide, cefminox, biapenem, doripenem, metropenem and ertapenem were from Zhejiang Hisoar Pharm. & Chem. Co., Ltd., Taizhou, China) and filtered with a 0.45-mm membrane filter (organic membrane, BJ225-PT, Shanghai Jiapeng Science and Technology Ltd., Shanghai).

2.2. Sample preparation

The model compounds (uracil, adenosine, uridine, cytosine, cytidine and guanosine) were used for the performance and retention mechanism evaluation of click lysine column. The test compounds (cytosine, adenosine, guanosine, salicylic acid, orotic acid and procainamide) were used for the investigation of retention behavior. The probe organic acids (*p*-chlorosalicylic acid, *p*-aminobenzoic acid, sorbic acid, anisic acid, cinnamic acid, benzoic acid) were dissolved in MeOH/H_2O (1:1, v/v) for chromatography test. The solutions of cephalosporins (ceftazidime pentahydrate, cefotaxime sodium, cefradine, cefepime hydrochloride, cefazolin sodium, cefprozil, cefpiramide and cefminox) and carbapenems (biapenem, doripenem, metropenem and ertapenem) were all prepared by dissolve them in MeOH/H_2O (1:2) and stored in a refrigerator for analysis and preparation of mixed samples.

2.3. Preparation of click lysine stationary phase (CLSP)

Stating from L-Boc lysine 1, the free amino group in L-Boc lysine was transferred by an inexpensive and shelf stable diazotransfer reagent 2 (imidazole-1-sulfonyl azide hydrochloride, which was prepared in our lab by literature method) [52] to get the azido Boc lysine 3. The Boc group was deprotected with trifluoroacetic acid (TFA) as follows: 3 (crude, 4 g) was dissolved in 20 mL DCM, and 5 mL TFA was added, then the mixture was stirred at rt for 3–4 h. TLC showed that 3 was consumed out, all volatiles was removed by vacuum evaporation to afford the key intermediate L-azido lysine 4 (Fig. 1). In order to confirm the structure of L-azido lysine 4, 4 reacted with phenyl alkyne in the presence of Cu(I). To a mixture of 4 (172 mg, 1 mmol) and phenyl alkyne (112 mg, 1.1 mmol) in 10 mL MeOH, CuI (19 mg, 0.1 mmol) was added, the mixture was stirred at rt for 2 h, removal of all solvents left a residue which was purified by passing through a silica gel column to afford (*S*)-2-amino-6-(4-phenyl-1H-1,2,3-triazol-1-yl) hexanoic acid 7 (Fig. 2), ^{1}H NMR ($CDCl_3$), δ: 7.84 (d, 2H, J = 7.2), 7.77 (s, 1H), 7.34 (tri, 3H, J = 7.2, J = 7.6), 4.41 (tri, 2H, J = 7.2), 1.85–1.88 (m, 2H), 1.67–1.72 (m, 2H), 1.99–2.03 (m, 2H), 4.32 (m, 1H), 5.10 (br, 2H, —NH_2). The terminal alkyne-silica beads 5 was prepared by condensation of 3-isocyanatopropyl triethoxysilane with propargylamine in anhydrous DMF, and then directly polymerized with silica beads [53]. Finally, the click reaction between terminal alkyne-silica beads 5 and L-azido lysine 4 *via* CuAAC was as follows: a suspension of terminal alkyne-silica gel 5 (5.0 g), L-azido lysine 4(3.4 g, 20 mmol) and CuI (38 mg, 2 mmol) in 100 mL MeOH:H_2O (1:2, v/v) was stirred slowly at 40 °C for 40 h. The silica beads were collected by filtration and washed well with MeOH (300 mL), 10% EDTA (500 mL), water (300 mL, 50 °C), MeOH (200 mL), THF (200 mL), acetone (200 mL),

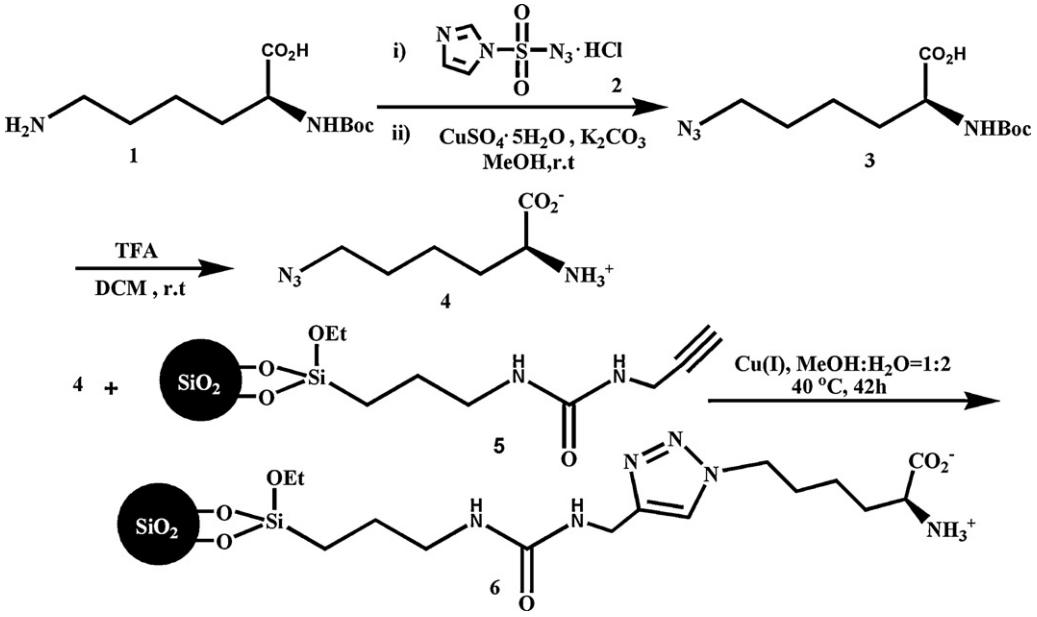

Fig. 1. The preparation of CLSP.

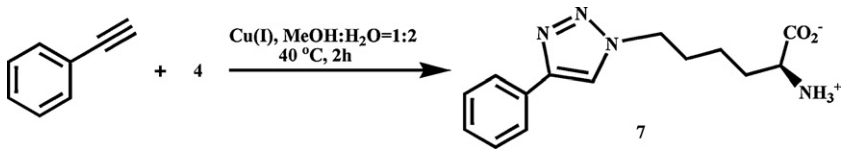

Fig. 2. Derivation of azido lysine for structure confirmation.

and DCM (200 mL) in turn. Finally, the silica beads were dried in vacuum to afford CLSP 6.

In order to display the advantage of click chemistry, we prepared tradition lysine stationary phase (TLSP) *via* literature method [54]. A mixture of L-Boc-lysine (3.69 g, 15 mmol) and dimethoxy(methyl)(3-(oxiran-2-ylmethoxy)propyl) silane (2.64 g, 12 mmol) in 60 mL dry DMF was stirred at 70–80 °C for 5 h. Then 4.0 g silica gel (activated by 10% HCl) was added in one pot. The heterogeneous suspension was stirred at 110 °C for 48 h. The silica beads was collected by filtration and treated with trifluoroacetic acid (8 mL trifluoroacetic acid in 50 mL MeOH) for 2 h, and the filter cake was washed with THF (200 mL), acetone (200 mL), MeOH (200 mL), H_2O (200 mL, 50 °C), MeOH (200 mL), DCM (200 mL) in turn. The silica was dried in vacuum before packing.

The lysine stationary phase prepared by above two methods was slurry-packed into stainless-steel columns (150 mm × 4.6 mm i.d.) with MeOH as slurry solvent and propulsion solvent respectively for the study of chromatographic behavior.

2.4. Apparatus and chromatographic conditions

FT-IR measurements were performed on a Nicolet 5SXC (Thermo Nicolet Corporation, Madison, WI, USA). ^{1}H NMR was carried out on a Bruker 400 (Bruker, Karlsruhe, Germany). Elemental analysis was measured on an elemental Vario EL III (Elementar Analysensysteme GmbH, Goettingen, Germany).

An Agilent (Agilent, Palo Alto, CA, USA) 1100 HPLC system consisting of a binary pump, a degasser, an auto sampler, an automatic thermostatic column compartment and a diode array detector (DAD) were used to perform the chromatographic experiments. Click lysine column (5 μm, 150 × 4.6 mm, i.d, home-made), tradition lysine (5 μm, 150 × 4.6 mm, i.d., home-made); Atlantics HILIC (5 μm, 150 × 4.6 mm, i.d., Waters, Milford, MA, USA); ZIC-HILIC (5 μm, 150 × 4.6 mm, i.d., Merck, Sequant, Sweden).

3. Results and discussion

3.1. Preparation and characterization of CLSP

The terminal alkyne-silica and CLSP were characterized by FT-IR and elemental analysis. The elemental analysis and the surface concentration are shown in Table 1. The increase of carbon content demonstrated that L-lysine was bonded to alkyne-silica successfully. According to the equation proposed by Kibbey and Meyerhoff [55], the surface concentration (calculated from nitrogen content) of alkynyl group on alkyne-silica was 2.73 μmol m^{-2}, and the surface concentration of CLSP (calculated from the nitrogen content) was 1.46 μmol m^{-2}, while the surface concentration of TLSP was 0.92 μmol m^{-2}.

Table 1
Elemental analysis of alkyne-silica and lysine linked stationary phases.

Stationary phase	C%	N%	Surface coverage (μmol m^{-2})
Alkyne-silica	8.29	2.29	2.73
Traditional lysine	9.09	0.77	0.92
Click lysine	10.49	4.73	1.46

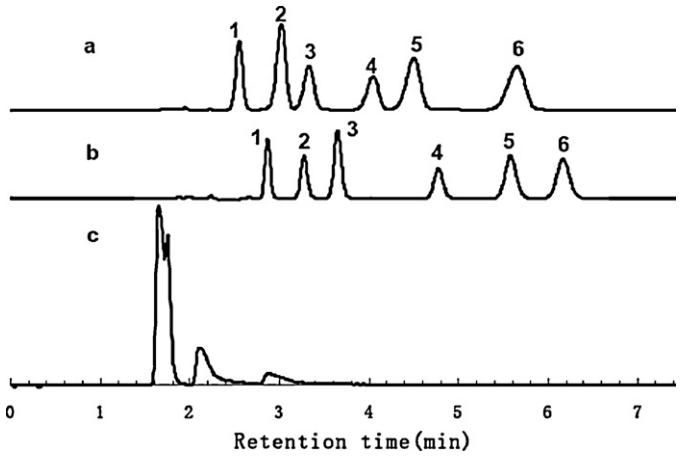

Fig. 3. Separation of nucleosides and bases on CLSP ((a) 5 μm, 150 mm × 4.6 mm i.d.), ZIC-HILIC ((b) 5 μm, 150 mm × 4.6 mm i.d.) and Atlantis HILIC-silica ((c) 5 μm, 150 mm × 4.6 mm i.d.). Conditions: flow rate 1 mL min^{-1}; 30 °C; mobile phase: acetonitrile:water (85:15, v/v); UV: 254 nm. Peak identification: (1) uracil, (2) adenosine, (3) uridine, (4) cytosine, (5) cytidine, and (6) guanosine.

3.2. Chromatographic evaluation under HILIC model

After the click lysine column was prepared, the evaluation of performance was carried out by using nucleosides and bases as probe compounds. As shown in Fig. 3, six nucleosides and bases were well separated with acetonitrile/water (85/15, v/v) as mobile phase. This new type of stationary phase showed good separation ability and selectivity, the mixed model compounds could be separated clearly in a short period without using any buffer, and good peak shapes are displayed, which is similar to the commercial ZIC-HILIC column, while commercial Atlantics could not separate these compounds at the same chromatography conditions. In addition, the symmetry factors of these compounds are quite good with appropriate retention. In the case of cytidine, the peak symmetry factor is 1.07, retention factor is 1.65, and the theory plate number was about 34,000 plates m^{-1} calculated according to the USP method. The Van Deemter plot [56] for cytidine on click lysine showed the optimal linear velocity was around 0.35 mm/s [Fig. 4].

For further understanding the chromatography behavior of click lysine, we carried out research on the relationship between

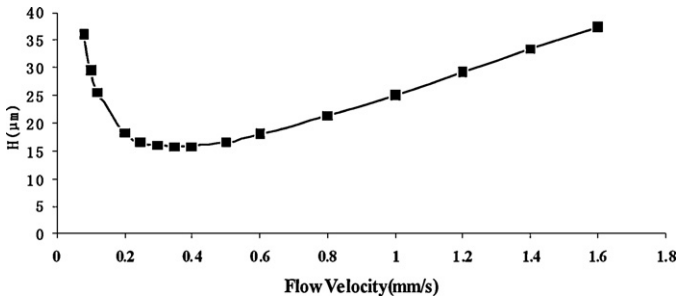

Fig. 4. Van Deemter plot for cytidine on CLSP (5 μm, 150 mm × 4.6 mm i.d.), conditions: column temperature, 30 °C; mobile phase: acetonitrile:water (85:15, v/v); UV: 254 nm.

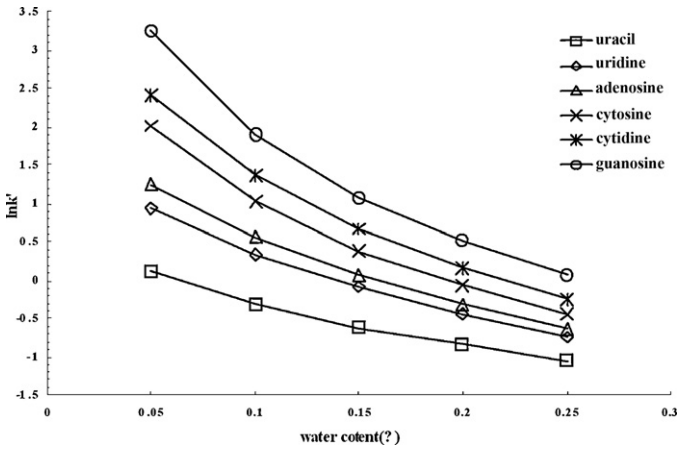

Fig. 5. The natural logarithmic plots of retention factors *k versus* water content ψ in mobile phase. Chromatographic conditions: flow rate 1 mL min^{-1}; 30 °C; mobile phase: acetonitrile/water (v/v) and water changed from 5% to 25% (v); UV: 254 nm. Model sample: uracil, adenosine, uridine, cytosine, cytidine and guanosine.

retention factors and the water content by using nucleosides and bases as probes, and the acetonitrile (ACN) in combined with water was used as eluent (water content ranges from 5% to 25%, v). The natural logarithmic of retention factors (K) was plotted *versus* the water content in the mobile phase. As shown in Fig. 5 click lysine exhibited typical HILIC characteristics, the retention time became shorter with the water content increase [57]. Furthermore, when we changed the content of ACN in mobile phase (ACN and water) from 10% to 90%, the retention of all analytes become shorter with the increase of water content in mobile phase, it exhibited typical HILIC characteristics. Some HILIC stationary phases were reported to display mixed-mode behavior [31,58,59], but in the case of CLSP, no critic ACN concentration was observed for mixed mode.

Both the performance evaluation and the study on the relationship between retention factor and water content showed that the click lysine is a typical HILIC stationary phase. Usually, the pH value and salt concentration may affect the retention of many analytes. In this case, the pK_a value of lysine is 2.18 and 8.95, but when the lysine was covalent bonded to silica, their pKa value may be changed. Herein, we subsequently investigated the retention of a wide range compounds (organic acids, nucleosides and bases as well as organic base) on click lysine under different pH values and salt concentrations. Firstly, 10 mM ammonium formate buffer solutions with pH ranging from 3.1 to 6.0 (adjusted by adding formic acid) were used to investigate the retention behaviors of click lysine under different pH values. As shown in Table 2, with the acidity of the eluent weakening (pH from 3.1 to 6.0), the retention times of salicylic acid and orotic acid were decreased, while the retention time of basic procainamide was increased. pH value plays an important role in separation of acidic or basic compounds on click lysine, and no obvious effect on their retention were observed on separation

Table 2
Retention time (min) of model compounds at different buffer pH values.[a]

Compounds	Retention time (min) at different buffer pH values		
	3.1	4.4	6.0
Cytosine	4.71	4.61	4.58
Adenosine	3.63	3.64	3.64
Guanosine	6.92	6.91	7.06
Salicylic acid	3.31	2.79	2.56
Orotic acid	7.80	5.84	5.33
Procainamide	3.11	4.78	5.40

[a] Mobile phase: ACN/10 mM ammonium formate (85/15, v/v). Column temperature: 30 °C. Flow rate: 1.0 mL min^{-1}. UV detection: 254 nm, t_0 = 1.7 min.

Table 3
Retention time of model compounds at different ammonium formate concentrations.[a]

Compounds	Retention time (min) at different ammonium formate concentrations		
	5 mM	10 mM	20 mM
Cytosine	4.58	4.60	4.87
Adenosine	3.56	3.60	3.75
Guanosine	6.86	7.07	7.70
Salicylic acid	4.46	3.55	3.14
Orotic acid	8.14	8.11	8.48
Procainamide	2.56	2.95	3.12

[a] Mobile phase: ACN/ammonium formate, pH = 3.1, (85/15, v/v) for model compounds. Column temperature: 30 °C. Flow rate: 1.0 mL min^{-1}. UV detection: 254 nm, t_0 = 1.7 min.

of nucleosides and bases. These facts could be explained such that the zwitterionic lysine containing both carboxylic acid and amine groups can be either protonated or deprotonated depending on the pH of the eluent, or that the zwitterionic solute orotic acid is substantially more polar at a pH where the carboxyl is not protonated, whereas the salicylic acid is weakly polar with both protonated and deprotonated carboxyl group. [60,61].

The salt concentration on retention was investigated by varying ammonium formate concentration from 5 to 20 mM (pH 3.1) in the mobile phase of acetonitrile/water (85/15, v/v). As shown in Table 3, little change on the retention time of nucleosides and bases was observed, for they were consistently charged at this pH, thus at buffer concentrations below 20 mM, the electrostatic effect was not applicable for nucleosides and bases. In additional, the buffer strength was not sufficient to promote elution, as it would be at a concentration above 20 mM. The minor increase in retention times for all solutes, with an increase in salt concentration to 20 mM, reflects the development of a sufficient buffer concentration to stabilize HILIC partitioning effects.

In order to broaden the applications of CLSP, it was then applied to separate organic acids using ACN:water (80:20, v:v) as mobile phase, and six organic acids were well separated (Fig. 6), though some of these peak shapes were not satisfied. When we tried to use different buffers for improving peak shape, such as ammonium formate (1.5 mM), ammonium acetate (0.3 mM) and potassium dihydrogen phosphate (0.75 mM) aqueous solution, no positive result was obtained, both the retention and separation efficiency were decreased. It should be noticed that no buffer was used in this case as well as in separation of nucleosides and bases, while eluent additives such as acids, bases or buffers were usually used either in

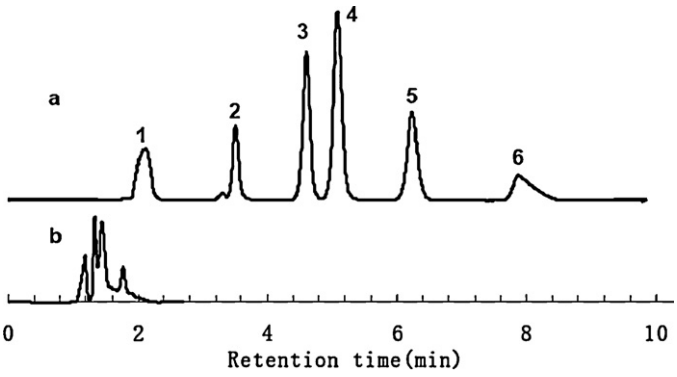

Fig. 6. the separation of six organic acids on CLSP ((a) 5 μm, 150 mm × 4.6 mm i.d.) and ZIC-HILIC ((b) 5 μm, 150 mm × 4.6 mm i.d.). Chromatography conditions: 80% ACN/20% H_2O (v/v), The column temperature: 30 °C. Flow rate: 1 mL min^{-1}. Wavelength: 254 nm. Sample: the mixed acids. Injection volume: 5 μL. Peak identification: (1) 4-chlorine salicylic acid, (2) 4-aminobenzoic acid, (3) sorbic acid, (4) anisic acid, (5) cinnamic acid, and (6) benzoic acid.

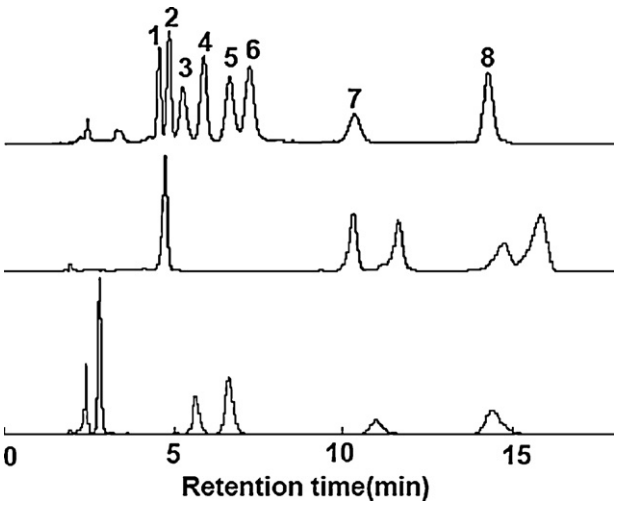

Fig. 7. Separation of eight cephalosporins on CLSP ((a) 5 μm, 150 mm × 4.6 mm i.d.), conditions: mobile phase: A, ACN/100 mM ammonium formate (90/10, v/v), pH = 4.5; B, 10 mM ammonium formate, pH = 4.5. Gradient eluent, 0–8 min, 88% A, 8–15 min, 88–70% A, 15–30 min, 70–30% A. The column temperature: 30 °C. Flow rate: 1 mL min^{-1}. Inject volume, 3 μL. Wavelength: 254 nm. Peak identification: (1) cefepime hydrochloride, (2) cefazolin sodium, (3) cefotaxime sodium, (4) cefpiramide, (5) cefprozil, (6) Cefradine, (7) cefminox, and (8) ceftazidime pentahydrate. The chromatographic conditions, such as mobile phase, column temperature, flow rate, injection volume and wavelength for TLSP and ZIC-HILIC were same as CLSP, except gradient eluent. TLSP ((b) 5 μm, 150 mm × 4.6 mm i.d.), gradient eluent: 0–20 min, 90–70% A, 21–30 min, 70–50% A, ZIC-HILIC ((c) 5 μm, 150 mm × 4.6 mm i.d.), gradient eluent: 0–15 min, 90% A, 15–0 min, 90–0% A.

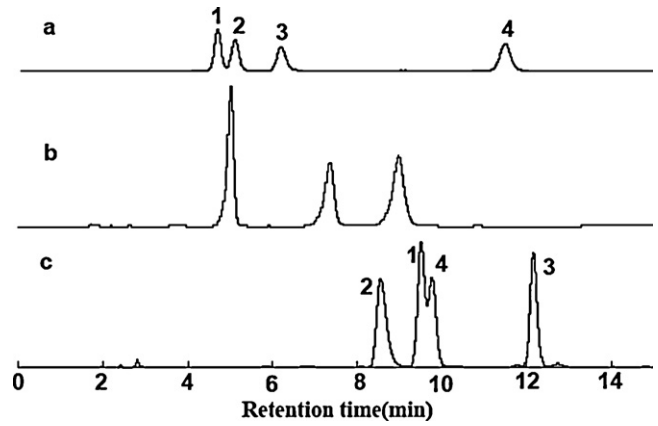

Fig. 8. Separation of four carbapenems on CLSP ((a) 5 μm, 150 mm × 4.6 mm i.d.), chromatography conditions: mobile phase A, ACN/100 mM ammonium formate (90/10), pH = 4.5. Mobile phase B, 10 mM ammonium formate, pH = 4.5. Gradient eluent, 0–5 min, 87% A, 5–10 min, 87–20% A. The column temperature: 30 °C. Flow rate: 1 mL min^{-1}. Sample: the mixed carbapenems. Inject volume, 1 μL. Wavelength: 254 nm. Peak identification: (1) biapenem, (2) doripenem, (3) metropenem, and (4) ertapenem. The chromatographic conditions, such as mobile phase, column temperature, flow rate, injection volume and wavelength for TLSP and ZIC-HILIC were same as CLSP, except gradient eluent. TLSP ((b) 5 μm, 150 mm × 4.6 mm i.d.), gradient eluent: 0–15 min, 90–85% A, 15–20 min, 85% A, and ZIC-HILIC ((c) 5 μm, 150 mm × 4.6 mm i.d.), gradient eluent: 0–5 min, 90% A, 5–10 min, 90–80% A, 10–15 min, 80% A.

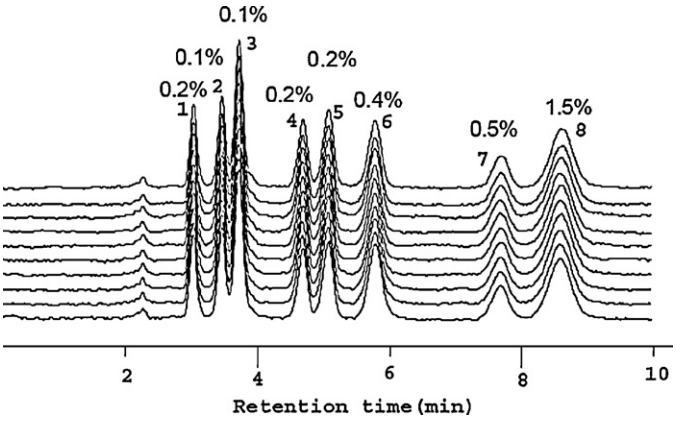

Fig. 9. Retention stability of eight cephalosporins on CLSP by continuously injecting ten times of the mixed cephalosporins. Mobile phase: acetonitrile/H_2O (85/15, v/v, 10 mM HCO_2NH_4, pH = 4.0) The column temperature: 30 °C. Flow rate: 1 mL min^{-1}. Inject volume, 1 μL. Wavelength: 254 nm. Peak identification: (1) cefepime hydrochloride, (2) cefradine, (3) cefprozil, (4) cefotaxime sodium, (5) cefazolin sodium, (6) cefpiramide, (7) cefminox, and (8) ceftazidime pentahydrate.

C18 or HILIC stationary phases for obtaining better peak shape and separation performance. This is a great advantage for application of this stationary in LC–MS combined technology. When commercial ZIC-HILIC was applied to separate these organic acids under same chromatographic conditions, weak retention and separation ability were observed.

3.3. Application in separating of cephalosporins and carbapenems

Cephalosporins and carbapenems are important antibiotics in pharmacy, many research and analysis requirements related to cephalosporins and carbapenems are gradually increased [62–70]. C18 was one of the most popular and powerful analysis stationary phases, and it was used to analyze cephalosporins and carbapenems. However, high water concentration (about 94%) mobile phases were usually used in C18 to afford appropriate retention and good peak shape due to the hydrophilicity of most of cephalosporins and carbapenems. Too high water content not only dramatically decreases the sensitivity of mass in LC–MS technology but also results in variable retention times in RPLC. HILIC is a good alternative for LC–MS due to the use of high content organic mobile phases in HILIC. Herein, eight cephalosporins and four carbapenems were selected as model compounds respectively, and we studied the application of click lysine on separating cephalosporins and carbapenems. As shown in Figs. 7 and 8, the cephalosporins and carbapenems were efficiently separated with good peak shapes and appropriate retention time. When commercial HILIC stationary phase (ZIC-HILIC) was applied to the analysis of cephalosporins and carbapenems at the optimized chromatographic conditions, inferior retention and separation ability were observed. Though TLSP exhibited better retention, its separation ability could not meet the requirement of analysis and separation.

3.4. The stability of CLSP

Chemical stability is an important parameter for stationary phases. The cephalosporins mixture was utilized for the investigation of the stability of CLSP through continuous ten injections (Fig. 9). The relative standard deviations (RSDs) of the first seven cephalosporins were all less than 0.5%, and the last peak of ceftazidime pentahydrate was about 1.5% for its wide peak and the result demonstrated a good stability of click lysine. In addition, the column was used for over 8 months without any deterioration, which further showed that the stationary phase can be widely and reliably used as separating material.

4. Conclusion

A zwitterionic HILIC click lysine column was prepared *via* click chemistry. This method showed high efficiency for bonding

functionalized zwitterionic groups. CLSP displayed good HILIC properties in the separation of nucleosides and bases. CLSP showed good stabilities under HILIC conditions and this type stationary may possess several retention mechanisms, such as partition mechanism, hydrogen bonding interaction and electrostatic interaction mechanisms. The effect of pH and salt concentration on retention were investigated for understanding its chromatographic properties. This new type of zwitterionic stationary could efficiently separate nucleosides and bases, as well as organic acids without using any buffer. The cephalosporins and carbapenems were also well separated under gradient eluent conditions. Compared with commercial HILIC (HILIC Atlantics) and TLSP, CLSP affords better retention and higher efficiency. The further investigations of CLSP on other application field have been undergoing in our laboratory.

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