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A novel amide stationary phase for hydrophilic interaction liquid chromatography and ion chromatography

Guobin Shen [a], FeiFang Zhang [a], Bingcheng Yang [a,*], Changhu Chu [a], Xinmiao Liang [a,b]

[a] School of Pharmacy, East China University of Science and Technology, Shanghai 200237, China
[b] Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

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ABSTRACT

A novel amide stationary phase (ASP) for hydrophilic interaction liquid chromatography (HILIC) has been prepared via the Click chemistry method. It was based on the strategy that the amino group of Asparagine was easily transferred to the corresponding azido group and then clicked onto terminal alkyne-silica gel in the presence of Cu(I)-based catalyst. For the tested polar compounds including nucleosides and nucleic acid bases, ASP-based column has demonstrated good performance in terms of separation efficiency and column stability, and the retention mechanism was found to match well the typical HILIC retention. In addition, the ASP described here showed much better selectivity in separation of inorganic anions under ion chromatography mode relative to other kinds of commercial ASP.

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1. Introduction

As a branch of high performance liquid chromatography (HPLC), hydrophilic interaction liquid chromatography (HILIC) has found many useful applications for separation of highly polar compounds that show no or little retention in conventional reverse phase C18 column. This interest had led to an accompanying increase of some new stationary phases (SPs). Up to now, many kinds of SPs for HILIC have been developed to suit specific separation problems [1]. The common HILIC SPs include bare silica, neutral polar groups (e.g., amide and diol), charged ligands (e.g. amino and sulfonate), and zwitterionic [2]. Among them, amide stationary phase (ASP) has been highly popular in HILIC [3]. In contrast to amino group, amide group does not possess basic property and is less reactive, so that the retention of ionizable analytes is not much affected by ion-exchange interactions. Irreversible sample adsorption is less probable, thus resulting in better stability over a long period of time [4]. Presently some HILIC ASPs have been commercially available, e.g. TSK-gel Amide-80 [5].

Click chemistry has been proved to be an efficient manner to introduce functional groups onto the surface of silica gel or other materials due to its high selectivity and efficiency [6,7]. By this method, some HILIC SPs with highly hydrophilic functional groups that are not easy to prepare via the conventional methods have been developed in recent years, e.g., maltose [8], cyclodextrin [8], oligoethylene glycol [9] and chitooligosaccharide [10], which have demonstrated good hydrophilic performance. In our previous work, several kinds of HILIC SPs have been described by transferring amino group of target molecule to azido group under very mild conditions via an efficient and shelf-stable diazotransfer reagent, imidazole-1-sulfonyl azide hydrochloride (ISAH), which was then clicked onto alkyne-silica gel [10–12]. These SPs showed good performance for the separation of polar compounds or for the enrichment of glycopeptides [10]. Up to now, such strategy has not been explored to prepare amide-based HILIC SP yet.

Recently some HILIC SPs have been found to behave well as anion exchanger [13–16], which may find extended applications in ion chromatography (IC). It is of interest to develop a SP with dual-function groups operated under both HILIC and IC [15]. Asparagine being a natural amino acid is easily obtained and it has two highly hydrophilic groups (amide and carboxyl). Starting from asparagine, we have described a novel amide-based HILIC SP by transferring the amino group of asparagine to the corresponding azido group via ISAH reagent, then clicked with terminal alkyne-silica gel (here it was called as Click-ASP). The preliminary performance of Click-ASP for HILIC and IC has been evaluated.

2. Experimental

2.1. Apparatus and reagents

Spherical silica was from Fuji (Fuji Silysia Chemical Ltd., Japan, 5 μm particle size; 10 nm pore size; 300 $m^2 g^{-1}$surface area).

* Corresponding author. Tel./fax: +86 21 64250622.
E-mail address: bcyang@ecust.edu.cn (B. Yang).

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Chromatographic grade acetonitrile, formic acid and ammonium formate were purchased from Tedia (USA). Ammonium formate and copper iodide were from Acros (USA). L-asparagine was obtained from Aladding Corp. (Shanghai, China). Other reagents were used without further purification. Milli-Q ultrapure water was used for solution preparation throughout.

The chromatographic experiments were performed on a Waters Alliance HPLC system consisting of a 2695 separation unit, automatic injector and column oven and a 2998-UV absorbance detector. Unless otherwise stated, 1 mL/min of the flow rate was used. Two kinds of ASP-based HILIC columns were used, including a homemade Click-ASP column (4.6 mm i.d. × 100 mm long; 5 μm dia.) and ACCHROM X-Amide column (4.6 mm i.d. × 150 mm long; 5 μm dia, ACCHROM, China).

FT-IR measurements were performed on a Nicolet 5SXC (USA). ^{1}H NMR identification was carried out on a Bruker 400 (Germany). Elemental analysis was measured on an elementar vario EL III (Germany).

2.2. Preparation of the Click-ASP and column packing

The preparation of terminal alkyne-silica gel and the diazotransfer reagent of ISAH was described previously [10,12]. The amino group of asparagine was firstly transferred to the corresponding azido group as follows. A mixture of asparagine (4.6 g, 30 mmol), K_2CO_3 (18 g, 130 mmol), $CuSO_4 \cdot 5H_2O$ (0.075 g, 0.3 mmol) and ISAH (8.5 g, 40.6 mmol) in 100 mL MeOH was stirred at room temperature for 10 h. The solution was then treated by rotary evaporation and vacuum filtration to yield brown-yellowish solid. The solid was resolved in methanol–HCl solution (prepared by dropping 20 mL acetyl chloride into 100 mL methanol in ice water bath), followed by reflux in oil bath at 64 °C for 11 h. The solution was cooled down and then treated by rotary evaporation to dryness to obtain yellow solid, which was then rinsed with anhydrous ethanol by rotary evaporation (100 mL × 3). The obtained solid was dissolved in pure water and extracted with ethyl acetate (100 mL × 3). The combined organic layer was dried via anhydrous Na_2SO_4. Evaporation of all volatiles afforded a light yellow solid (α-azido-Asparagine methyl). To get the key intermediate of α-azido-Asparagine, the hydrolysis of above α-azido-Asparagine methyl was performed as follows. In ice water bath, α-azido-Asparagine methyl was dissolved in 100 mL methanol and 2.245 g NaOH. When NaOH completely dissolved, the ice bath was removed and the solution was stirred under ambient temperature for 4 h to afford the key intermediate α-azido-Asparagine, followed by adding some amount of 50% acetic acid to adjust the pH value to 6.0 for further use.

The synthesis route of Click-ASP was provided in Fig. 1. Briefly, terminal alkyne silica gel (2.0 g) was added into above α-azido-Asparagine solution (1.7 g, 5.2 mmol) in 60 mL MeOH/H_2O (v/v, 1/2), then adding a Cu(I) catalyst which was newly prepared by mixing copper(II) acetate (0.4 g, 2 mmol) and sodium ascorbate (0.8 g, 4 mmol) in 5 mL water. This suspension was stirred slowly at 40 °C for 50 h. After filtration, the filter cake was washed with water (300 mL), 10% EDTA (300 mL), warm water (600 mL, 50 °C), acetone (300 mL), and tetrahydrofuran (50 mL) in turn. The product was then collected and dried in vacuum before packing. The Click-ASP was slurry-packed into stainless-steel column (4.6 mm i.d. × 100 mm length) with methanol as slurry solvent and propulsion solvent.

3. Results and discussion

3.1. Characterization of Click-ASP

α-azido-Asparagine methyl was characterized by FTIR and ^{1}H-NMR (see Support Information SI-Fig. 1), respectively. An obvious absorption peak at 2115 cm^{-1} corresponding to N_3 group was observed by IR spectrum, indicating that the amino group of asparagine was successfully transferred to azido group. The alkyne-silica gel and Click-ASP was characterized by FTIR and the elemental analysis, as provided in Support Information of SI-Fig. 2 and SI-Table. The peak corresponding to azide group at 2115 cm^{-1} for α-azido-Asparagine methyl disappeared after clicking step. In comparison with alkyne-silica gel, an increase of carbon and nitrogen content of Click-ASP was observed, indicating that α-azido-Asparagine was successfully bonded to alkyne-silica gel. The apparent anion exchange capacity of Click-ASP was determined to be ~2.55 mmol/g from the average nitrogen percentage obtained by the elemental analysis data (*N*%=2.09). It was lower than that of Click-lysine SP (*N*%=4.73) [12] and slightly higher than Click-amino SP (*N*%=2.02) [15] previously described.

3.2. Chromatographic evaluation of Click-ASP under HILIC mode

The performance of Click-ASP operated under HILIC mode was explored to separate several nucleosides and nucleic acid bases, including uracil, adenine, cytosine, cytidine and guanosine. A typical chromatogram was provided in Fig. 2. It can be seen that several nucleosides could be well separated in less than 10 min under isocratic mode. The elution order was consistent with their hydrophilic degree. The calculated theoretical plate numbers for these analytes were in the range of 27,000 to 35,000/m. Although slight tailing peaks were observed, which probably resulted from packing process, the peak asymmetry factors ranging from 1.56 to 1.69 could still be accepted. For Click-ASP column, the plots between the linear velocity and the theoretical plate height were fitted well with the Van–Deemter curve for adenine and cytidine, respectively, as shown in the Support Information of SI-Fig. 3. The linear velocities corresponding to the minimum plate height were~0.5 mm/s.

As mentioned above, a desirable feature of Click-ASP is its high running stability relative to amino-based ones owing to less reactivity

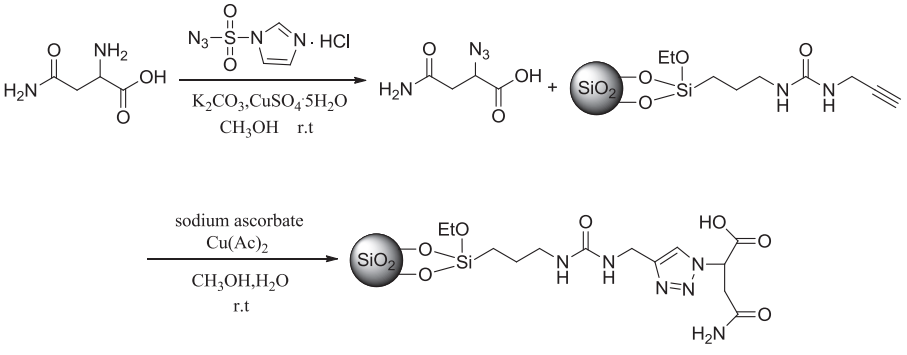

Fig. 1. Synthesis diagram of Click-ASP.

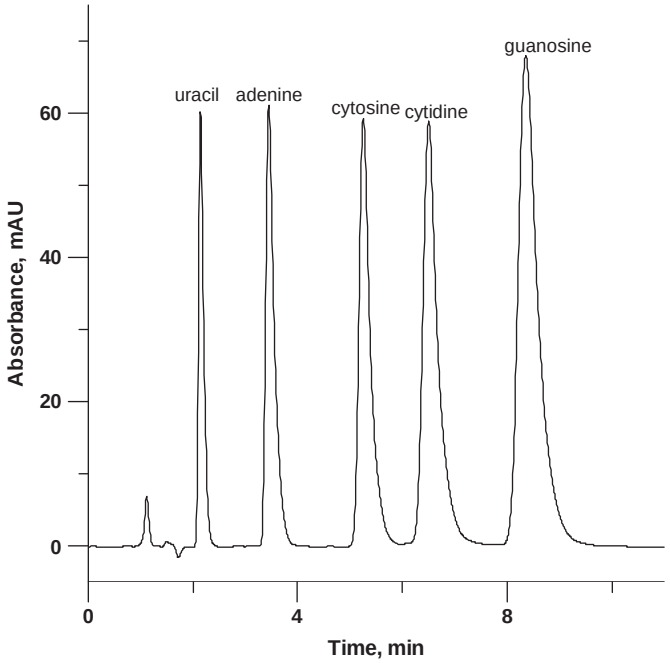

Fig. 2. Chromatogram of separation of nucleosides and nucleic acid bases by Click-ASP column. Conditions: column, 4.6 mm i.d. × 100 mm long; mobile phase, 90% ACN/10% H_2O, 100 mM $HCOONH_4$, pH = 3.06; absorbance wavelength, 254 nm; flow rate, 1.0 mL/min; injection volume, 5 μL; column temperature, 30 °C.

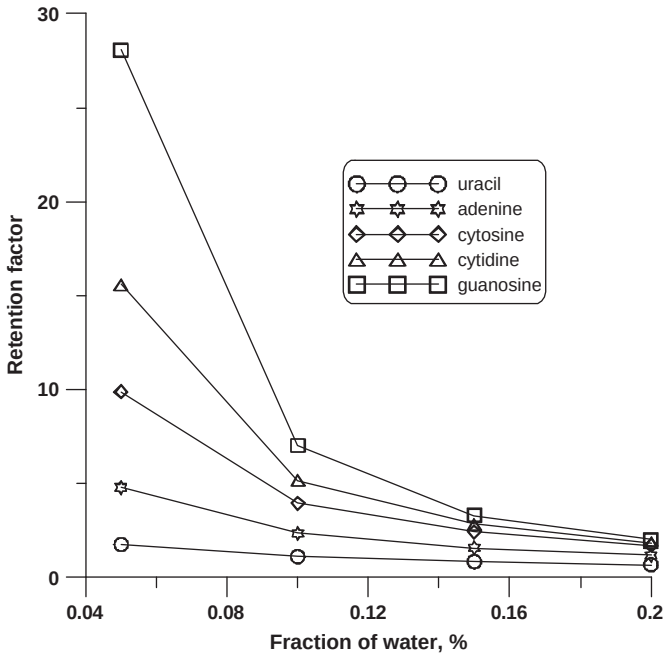

Fig. 3. Effect of water content in the mobile phase on the retention of analytes. Other conditions same to Fig. 2.

of the amide group. Herein, the operation stability of Click-ASP column was evaluated by separating nucleoside mixture through ten consecutive injections, as shown in the Support Information of SI-Fig. 4. The relative standard deviations (RSDs) of retention times and peak areas of several tested analytes were all less than 0.08% and 1%, respectively. Inter-day reproducibility of retention factors of target analytes in 7 days was also explored and RSDs were less than 0.8%. In addition, no obvious change of the retention factors after one month continuous use was observed. These revealed rather good stability of Click-ASP column.

In addition, the separation of small molecule organic bases was also performed on Click-ASP column. Baseline separation of four organic bases could be achieved (data not shown). Less retention of fours organic bases tested was observed, which probably resulted from the electrostatic repulsion interaction between the protonated bases and 1,2,3-triazole ring existing onto the surface of Click-ASP for the eluent operated at low pH value (3.06).

To examine the separation mechanism of Click-ASP, the retention behavior of nucleosides was investigated by varying the water content in the mobile phase (acetonitrile/H_2O). The plot of the retention factors of model analytes and water content in the mobile phase was shown in Fig. 3. The retention of all analytes tested decreased with the increase of water content in the mobile phase, which followed the typical HILIC separation mechanism. That is to say, there is a diffuse layer containing increased content of aqueous phase at the SP surface under HILIC conditions for retention of polar analytes [17].

The presence of electrolyte in the mobile phase is known to have an impact on separations under HILIC mode. Herein such effect was explored by using ammonium formate as additive electrolyte, as provided in the Supplementary Material of SI-Fig. 5. The general trend was that the retention of all analytes increased with the increase of electrolyte concentration, which matched well the typical HILIC behavior since the increase of electrolyte in the mobile phase would lead to the enhancement of the polarity of the rich aqueous layer over the SP surface. Such effect is especially pronounced for the strongly hydrophilic analytes. Additionally, improved separation selectivity was observed with the increase of electrolyte concentration. Obviously, the typical HILIC mechanism dominates the separation process. The effect of pH value of the mobile phase on the separation of nucleosides was also explored, as provided in the Support Information of SI-Fig. 6. In the tested pH range of 3.0–5.4, much less effect of pH value of the mobile phase on the retention factor was observed.

To demonstrate the effect of Click-ASP, comparison with commercial amide-based HILIC column was made under the same condition. The achieved chromatogram was provided in the Support Information of SI-Fig. 7. Baseline separation of several tested analytes could be achieved on both columns. In consideration of the different length of two columns (100 mm & 150 mm), the retention of the analytes tested on two columns was almost same (e.g., the ratio of retention factor of the third and the fifth peak was 0.979 and 0.983, respectively), indicating good performance of Click-ASP column.

3.3. Chromatographic evaluation of Click-ASP under IC mode

The existence of 1,2,3-triazole group introduced in the Click reaction renders its possibility to behave anion exchange sites, as proved previously [15,18]. The potential ability of Click-ASP for IC was evaluated by separating several inorganic anions with UV absorption with the eluent of 5 mM Na_2SO_4 with the addition of small amount of H_2SO_4, as provided in Fig. 4. Five inorganic anions including IO_3^-, BrO_3^-, Br^-, NO_3^- and I^- were well separated in less than 4 min. The ion exchange mechanism was responsible for such behavior, proved by the fact that the retention times of the analytes on Click-ASP decreased with the increase of the eluent concentration. The relative standard deviation (RSD) of retention times (and peak area) of above analytes over 13 consecutive runs were 0.18% (2.28%), 0.19% (1.61%), 0.18% (1.49%), 0.17% (1.47%), 0.18% (2.35%), respectively, indicating rather good run-to-run reproducibility of Click-ASP (see Supplementary Material in SI-Fig. 8). By comparison, other two commercial amide-based columns prepared by common method demonstrated no or poor separation ability for above anions. This mainly resulted from the existence of

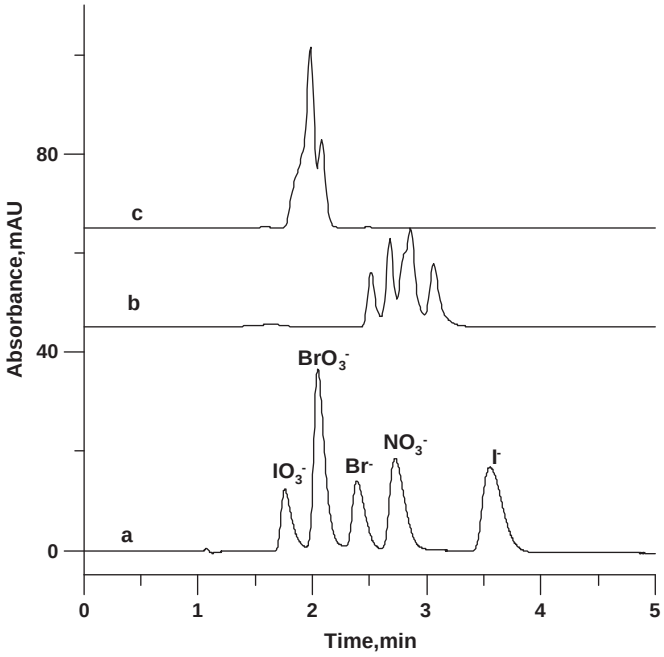

Fig. 4. Inorganic anion separation by three kinds of amide-based columns. Conditions: a, Click-ASP column; b, ACCHROM X Amide-based column; c, TSK Amide-80; eluent, 5 mM Na_2SO_4+0.5 mM H_2SO_4; injection volume, 5 μL; UV absorbance wavelength, 210 nm; flow rate, 1.0 mL/min.

1,2,3-triazole ring onto the surface of Click-ASP which render it more selective sites compared to sole amide group.

4. Conclusion

A novel amide-based HILIC SP was prepared via clicking asparagine on azide-silica gel. The SP has very simple structure and is easy to be prepared. The SP-based column demonstrated good separation performance for both HILIC and IC mode. In consideration of its good stability, it believes it will find useful applications. Such strategy can be also used for preparation of similar amide-based HILIC SP.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.04.046.

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