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Short communication

Analysis of cephalosporins by hydrophilic interaction chromatography

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ABSTRACT

A simple hydrophilic interaction chromatography (HILIC) method was developed to analyze seven cephalosporins. These seven cephalosporins could be separated well on the Click β-CD column and Atlantis HILIC Silica column. The effects of buffer concentration and pH on the retention under HILIC mode were studied. Except cefepime hydrochloride (**4**), the retention of other six cephalosporins increased with increasing buffer concentration, while decreased with increasing pH. Different separation selectivities could be observed on the Click β-CD column and Atlantis HILIC Silica column, and changing pH also resulted in the changing of separation selectivity. The separations of cephalosporins by HILIC and reversed-phase high performance liquid chromatography (RP-HPLC) were compared, and the two separation modes had good orthogonality. In addition, cefotaxime sodium (**1**) and its degradation were separated well on the Click β-CD column, which indicated that the Click β-CD column by HILIC can be used for studying the stability of cephalosporins.

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1. Introduction

Cephalosporins can treat infections caused by Gram positive and Gram negative bacteria by interfering the formation of bacterial cell wall. They are the most frequently used antibiotics due to their broad antibacterial spectrum. More than 60 cephalosporins in four generations have already been available in the market, so it is important to develop analysis methods for their quality control. The analysis of cephalosporins is not only limited in pharmaceutical analysis, but also extent to food safety and environmental protection, such as monitoring antibiotic residues in milk, edible tissues of animals [1] and wastewater from butcheries or hospitals [2]. All these analytical tasks need high performance separation.

Cephalosporins are semi-synthetic antibiotics derived from 7-aminocephalosporanic acid including a dihydrothiazine ring and a β-lactam ring. There is at least one carboxyl group in the structure, and some cephalosporins possess zwitterionic structure. In the last few decades, RP-HPLC has been the most widely used method for the analysis of cephalosporins [3]. With this method, the buffer, acid and ion-pair are often used as additives [4–9]. Ion-pair, such as, tetrabutylammonium hydroxide can partially neutralize the charged analytes, so that both the retention and peak shape of these analytes can be improved [8]. In addition, multidimensional HPLC system has been developed to analyze cephalosporins. Nishino et al. used two dimensional HPLC system with coupled ion-exchange and RP columns to separate cefmatilen hydrochloride hydrate and its metabolites in plasma and urine [10]. Capillary HPLC is also used to analyze cephalosporins at lower concentration [11,12], which is especially suitable for the trace determination of cephalosporins in environmental and food samples. Besides the HPLC method, cephalosporins can also be analyzed by capillary electrophoresis [13] and ion-exchange planar electrochromatography [14].

In 1990, A. J. Alpert firstly proposed HILIC which is particularly promising for the separation of polar compounds [15]. With the development of HILIC, it has been widely used in many fields, such as metabonomic study [16], analysis of pharmaceutical and their impurities [17] and two dimensional (2D)-LC analysis [18]. It is reported that HILIC was also used to analyze cephalosporin C [19,20]. The feasible mobile phase of HILIC and its compatibility with MS open a new door for the analysis of cephalosporins. In this paper, HILIC was developed to separate seven commonly used cephalosporins. Column, buffer concentration and pH were investigated to illustrate their effects on the retention and separation selectivity of cephalosporins. The orthogonality between HILIC mode and RP-LC mode for cephalosporins was also investigated. Furthermore, a successful HILIC method was developed to analyze cefotaxime sodium (**1**) and its degradation products.

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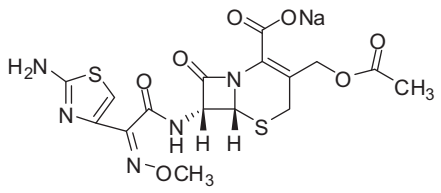
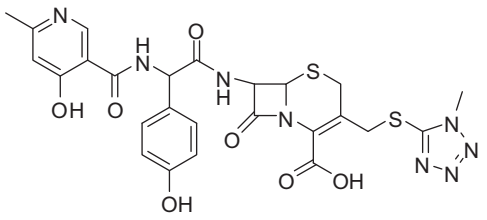
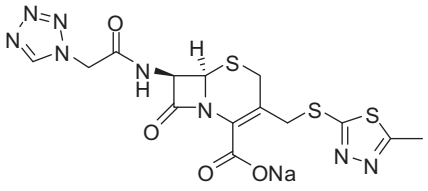


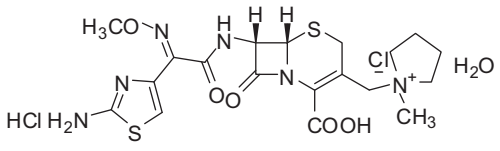
cefotaxime sodium (1)



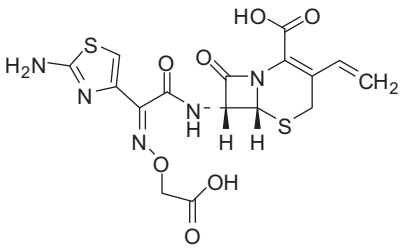
cefpiramide (2)



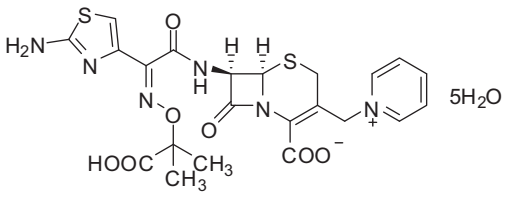
cefazolin sodium (3)



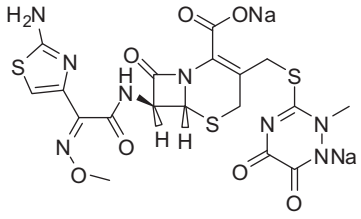
cefepime hydrochloride (4)



cefixime (5)



ceftazidime (6)



ceftriaxone sodium (7)

Fig. 1. Chemical structures of seven cephalosporins.

2. Experimental

2.1. Reagents and materials

The raw materials of seven cephalosporins were gifts from northeast general pharmaceutical factory. And the structures of them are shown in Fig. 1. Formic acid (98% pure) was purchased from Acros (USA), and ammonium formate (analytical-reagent grade) was purchased from Aladdin (China). HPLC grade acetonitrile was purchased from TEDIA (USA). The water used in this study was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Instruments

All the chromatographic separations were performed on an Agilent 1200 HPLC system (Agilent, USA), which comprised G1312B binary pump, G1379B degasser, G1367C autosampler, G1316B thermostatic column compartment and G1315C diode array detector (DAD).

The mass spectrometry (MS) determination was performed on Waters ACQUITY UPLC™ system with a Quattro Micro MS (triple quadrupole) operating in ESI^+ mode (Waters, USA).

2.3. Chromatographic and MS conditions

The columns used under HILIC mode were the Click β-CD column [21] (150 mm × 2.1 mm i.d., 5 μm, 10 nm pore size, home made) and Atlantis HILIC Silica column (100 mm × 2.1 mm i.d., 5 μm, 10 nm pore size, Waters, USA). An XTerra MS C18 column (150 mm × 2.1 mm i.d., 5 μm, 12.5 nm pore size, Waters, USA) was used under RP-HPLC mode. All HPLC experiments were done under the following conditions: Flow rate was 0.2 mL/min. Column temperature was controlled at 30 °C. 254 nm was chosen as the detection wavelength. The mobile phase was filtrated with microp-

orous membranes (0.22 μ). The detailed mobile phase and gradient condition were listed in the figures.

ESI-MS and MS^2 parameters were as follows: capillary voltage is 3.0 kV, cone voltage is 20 V, nitrogen was used as the desolvation gas at 600 L/h. Source temperature and desolvation temperature is 120 °C and 380 °C, respectively. Argon was employed as collision gas, and collision energy is 20 V to obtain MS^2 data.

2.4. Sample preparation

Cefotaxime sodium (**1**), cefazolin sodium (**3**), cefepime hydrochloride (**4**), ceftazidime (**6**) and ceftriaxone sodium (**7**) were prepared at 1 mg/mL in MeOH–H_2O (50/50, v/v). Cefpiramide (**2**) and cefixime (**5**) were prepared at 1 mg/mL in MeOH. The mixed samples were prepared at 1 mg/mL for each cephalosporin. All the samples were filtrated with microporous membranes (0.22 μ). Because of the instability of the cephalosporins, the samples were stored at 4 °C to prevent the degradation.

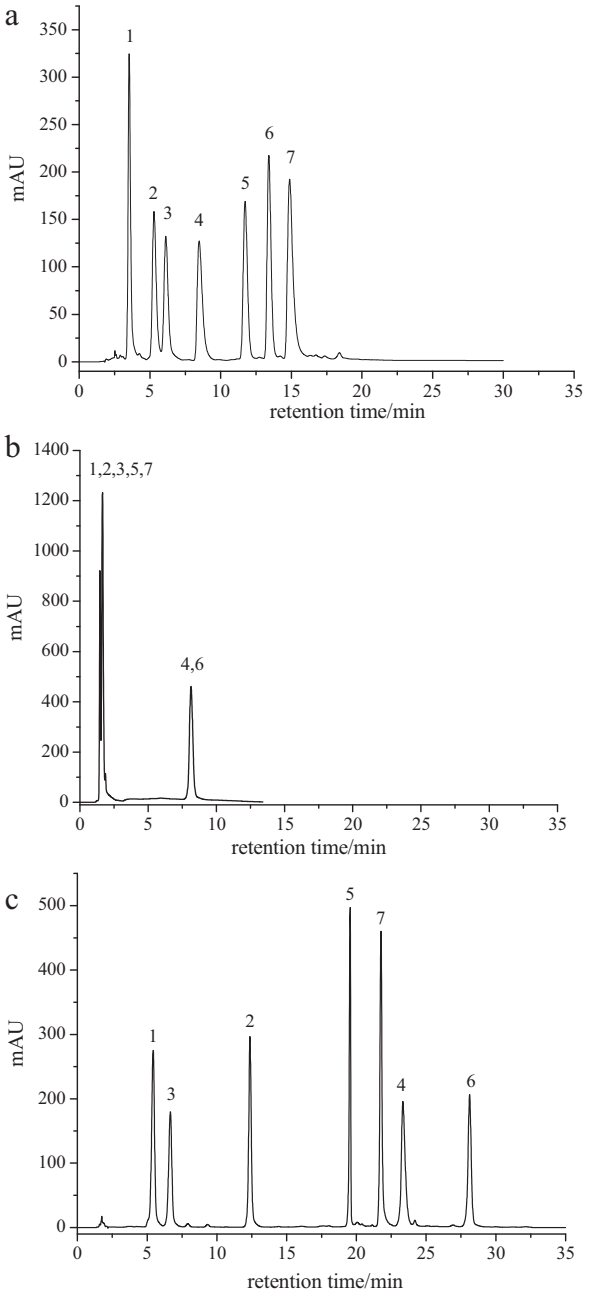


Fig. 2. Chromatograms for seven cephalosporins on the Click β-CD column (a) and Atalantis HILIC Silica column (b, c). Mobile phase: A, 10 mM ammonium formate at pH 6.8; B, acetonitrile/100 mM ammonium formate = 90/10 at pH 6.8. Gradient for (a) and (b) was 88%–65%B in 20 min, and 65%B in the next 10 min; gradient for (c) was 100%–75%B in 20 min, and 75%B in the next 15 min. (**1**) Cefotaxime sodium; (**2**) cefpiramide; (**3**) cefazolin sodium; (**4**), cefepime hydrochloride; (**5**), cefixime; (**6**), ceftazidime; (**7**), ceftriaxone sodium.

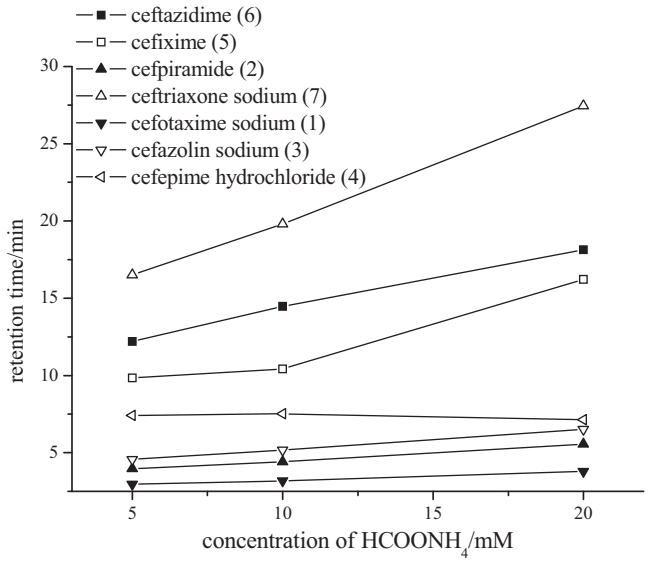


Fig. 3. Effect of buffer concentration on the retention of seven cephalosporins on the Click β-CD column. Mobile phase: ammonium formate (concentration as noted, pH = 6.8), with 76.5% ACN.

2.5. Method validation

The cefazolin standard and cefazolin sodium injection were prepared in MeOH–20 mM ammonium formate (50/50, v/v). In the linearity experiment, the cefazolin standard was prepared at five concentration levels, 60 μg/mL, 80 μg/mL, 100 μg/mL, 120 μg/mL, 140 μg/mL. In the recovery experiment, quality control samples were prepared by spiking the cefazolin standard (80%, 100%, 120% of the known concentration of cefazolin sodium injection) in the

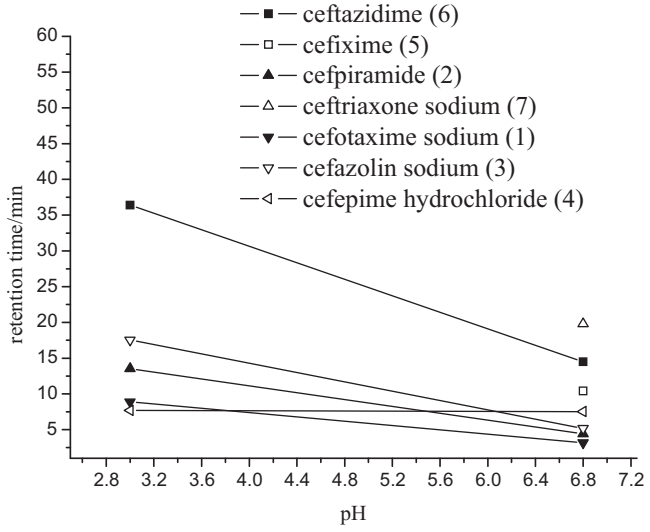


Fig. 4. Effect of pH on the retention of seven cephalosporins on the Click β-CD column. Mobile phase: ammonium formate (10 mM, pH value as noted), with 76.5% ACN. Ceftriaxone sodium (**7**) and cefixime (**5**) were not eluted within 60 min at pH 3.0.

cefazolin sodium injection solution (50 μg/mL) to obtain 3 different concentration levels within the calibration curve range, and three samples were prepared at each concentration level. In the reproducibility experiment, six samples of cefazolin sodium injections were prepared at the concentration of 100 μg/mL. The chromatographic conditions were as follows, mobile phase: A, 10 mM ammonium formate at pH 6.8; B, acetonitrile/100 mM ammonium formate = 90/10 at pH 6.8. Gradient was 90–60%B in 20 min.

3. Results and discussion

The standard calibration curve (concentration as a function of chromatographic peak area) for cefazolin were linear (R^2 = 0.9998, n = 5) over the concentration range, 60–140 μg/mL. And the LOD and LOQ for cefazolin sodium, which were established as the amounts for which the signal-to-noise ratios were 3:1 and 10:1, respectively, were 0.44 μg/mL and 1.47 μg/mL, respectively. To confirm the precision, the standard at the concentration of 100 μg/mL was analyzed six times, and the RSD was 0.18%. To confirm the accuracy of the proposed method, recovery experiments were carried out by standard addition technique. The average recovery was 100.57%, and the RSD value was 1.39%. To confirm the reproducibility, six samples of cefazolin sodium injection at the concentration of 100 μg/mL were analyzed. The average content was 94.76%, and the RSD was 0.44%.

In this work, seven cephalosporins can be separated well by HILIC. This method is convenient not only to study the effect factors on the separation of them by HILIC, but also to monitor more than one kind of cephalosporin rapidly in wastewater, milk and so on. Firstly, separations of these seven cephalosporins on the Click β-CD column and Atlantis HILIC Silica column were compared. When the pH of the eluent was 6.8, the retention of these seven cephalosporins on the Click β-CD column was longer than that on Atlantis HILIC silica column under the same condition (Fig. 2a and b). On the Click β-CD column, these seven cephalosporins could be separated well under this condition, while five cephalosporins (compounds **1**, **2**, **3**, **5** and **7**) were nearly eluted at dead time on Atlantis HILIC silica column, and the other two cephalosporins (compounds **4** and **6**) were more retained. The reason may be that compounds **4** and **6** are less acidic than the other cephalosporins, and less repulsive interactions between silanol surface and solutes might occur or even cation exchange at pH 6.8. After optimizing the gradient condition, these seven cephalosporins could be separated well on Atlantis HILIC silica column (Fig. 2c). However, these seven cephalosporins had extremely different elution orders on the two HILIC columns. For the difference of retention time, the reason is probably that the Click β-CD column has more hydroxyl groups, and its hydrophilicity may be stronger than that of Atlantis HILIC silica column. Furthermore, other interactions, such

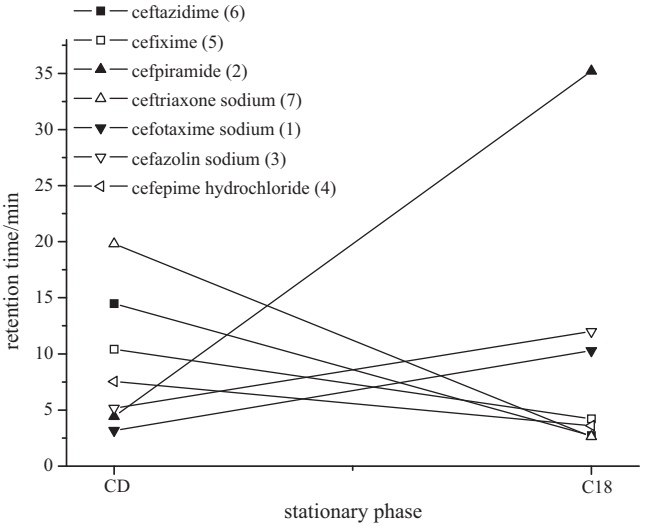


Fig. 5. The comparison of the retention time of seven cephalosporins on the Click β-CD column and C18 column. Mobile phase: A, 10 mM ammonium formate; B, acetonitrile/100 mM ammonium formate (90/10). 85%B was used on the Click β-CD column and 10%B was used on C18 column.

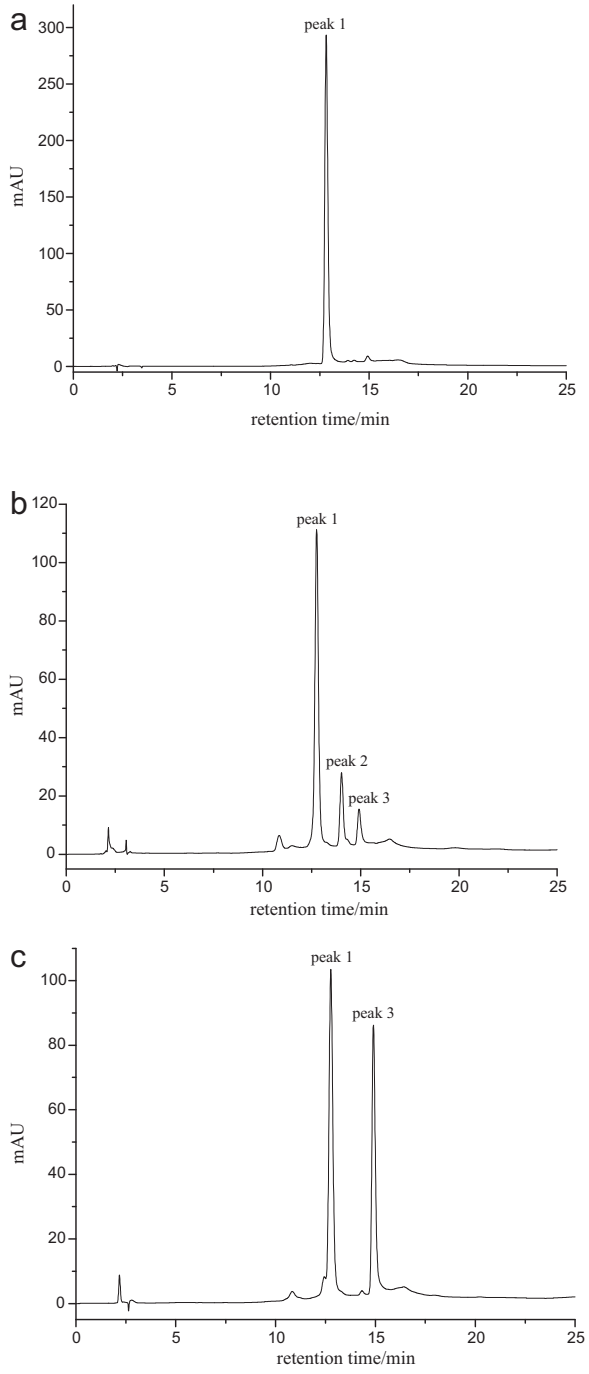


Fig. 6. Chromatograms of cefotaxime sodium after being treated under the following conditions. (a) Cefotaxime sodium in MeOH–H_2O (50/50, v/v); (b) cefotaxime sodium in MeOH–H_2O (50/50, v/v) at 70 °C for 1 h; (c) cefotaxime sodium in H_2O at 70 °C for 1 h. Peak 1 represents cefotaxime sodium (**1**); peak 2 and peak 3 represent degradation compounds. Mobile phase was the same as noted in Fig. 2. Gradient for the three chromatograms was 100–70%B in 15 min, and 70%B in the next 10 min. Column: the Click β-CD column.

as ion-exchange, electrostatic repulsion effects resulting from the triazole ring on the Click β-CD material [21] might be involved in the separation, which leads to the orthogonality of the two columns.

The effect of buffer concentration on the retention and selectivity of these seven cephalosporins was investigated at 5 mM, 10 mM, 20 mM of ammonium formate (Fig. 3). With the buffer concentration increasing, these cephalosporins had better retention on the Click β-CD column except cefepime hydrochloride (**4**). However, the buffer concentration could not change the elution order of these cephalosporins. It is reported that the retention time of organic acids increased with the increasing of buffer concentration by HILIC, while the retention time of alkaloid decreased with the increasing of buffer concentration [21]. Cefepime hydrochloride (**4**) possesses a quaternary ammonium cation and a primary amine but only one carboxyl group, which makes it more basic than other six cephalosporins. So the retention time of cefepime hydrochloride (**4**) decreased with the increase of buffer concentration. It is worthwhile to mention that the salt concentration has different effects on different solutes, e.g. it was reported that retention of thirteen dipeptides was inversely proportional to the concentration of triethylamine phosphate at pH 2.8 [15].

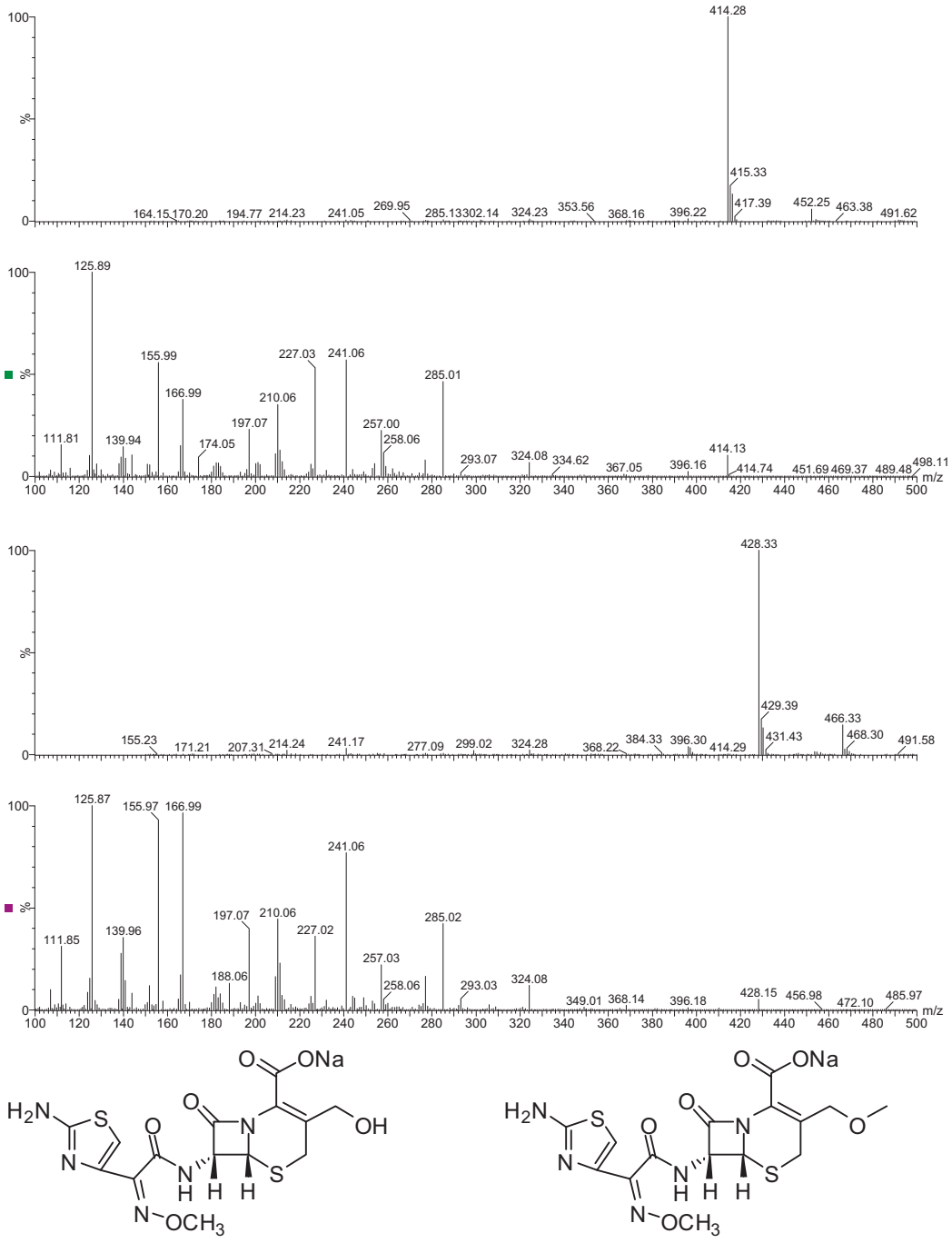


Fig. 7. The ESI-MS and MS^2 chromatograms of the degradation compounds of cefotaxime sodium. (a) and (b) ESI-MS and MS^2 of the ion at *m/z* 414 spectra of peak 3; (c) and (d) ESI-MS and MS^2 of the ion at *m/z* 428 spectra of peak 2.

A further separation at the two pH was examined to investigate the influence of pH on retention and separation selectivity (Fig. 4). When pH was 3.0, these cephalosporins showed strong retention on Click β-CD column, and even two cephalosporins (ceftriaxone sodium (**7**) and cefixime (**5**)) could not be eluted within 60 min. Ceftriaxone sodium (**7**) contains a strongly acidic, heterocyclic system [22], and cefixime (**5**) contains two carboxyl groups. These acidic groups might interact strongly with the residue hydroxyl groups and protonated triazoles on the Click β-CD column at low pH [21]. Ceftazidime (**6**) also has two carboxyl groups, but it possesses a quaternary ammonium group which had electrostatic repulsion interaction with triazole rings on the Click β-CD material. The additive effects of ion-exchange and electrostatic repulsion made ceftazidime (**6**) eluted within 60 min but with much stronger retention than other four cephalosporins. When pH was increased to 6.8, the retention of all cephalosporins except cefepime hydrochloride (**4**) decreased, and the possible reasons may be the decreasing of ion-exchange effect. Additionally, different elution order could be observed at the two pH (Fig. 4). For example, cefotaxime sodium (**1**) and cefepime hydrochloride (**4**) were eluted almost at the same time at pH 3.0, but these two cephalosporins could be separated well at pH 6.8 and the eluted order was just opposite to that at pH 3.0.

Cephalosporins can be separated by both HILIC and RP-LC modes. The separation selectivity by the two modes was also investigated on the Click β-CD column and XTerra MS C18 column, and there is marked difference in elution order of these seven cephalosporins between the two modes (Fig. 5). Cefazolin sodium (**3**), cefotaxime sodium (**1**) and cefpiramide (**2**) have better retention on C18 column, but their retention on the Click β-CD column was relatively weak. The other four cephalosporins have weak retention on C18 column, but they could be separated well on the Click β-CD column. Good orthogonality between the two modes may be helpful for the analysis of impurities in cephalosporins.

A primary experiment was carried out to analyze the degradation compounds of cephalosporins by HILIC. Cephalosporins are not stable, especially at high temperature. In this experiment, cefotaxime sodium (**1**) was heated at 70 °C in the solvents of MeOH–H_2O (50/50, v/v) and H_2O respectively for 1 h and then analyzed by HILIC on the Click β-CD column. From Fig. 6, it can be seen that solvents also had great influence on the stability of cefotaxime sodium (**1**). In the solvent of MeOH–H_2O (50/50, v/v), cefotaxime sodium (**1**) was obviously degradated into peak 2 and peak 3 after being heated (Fig. 6b), and in the solvent of H_2O, cefotaxime sodium (**1**) (peak 1) was mainly degradated into peak 3 (Fig. 6c). Peak 2 and peak 3 were further identified by MS and MS^2. The $[M+H]^+$ of peak 3 was 414, and the characteristic fragment ions at m/z 285, 241(cleavage of the β-lactam ring), 197 and 167 (Fig. 7b) were compared with those data reported [23]. And the result indicated peak 3 was deacetylcefotaxime sodium. The $[M+H]^+$ of peak 2 was 428, showing an increase of 14 Da, and its MS^2 spectra (Fig. 7d) presented very similar to that of deacetylcefotaxime sodium. So peak 2 was firstly identified as cefpodoxime sodium. In the solvent of H_2O, cefotaxime sodium (**1**) (peak 1) was easily hydrolyzed into deacetylcefotaxime sodium (peak 3), but in the solvent of MeOH–H_2O (50/50, v/v), MeOH may react with deacetylcefotaxime sodium (peak 3) obtaining cefpodoxime sodium (peak 2). The result suggests that it is important to choose solvents when doing experiments about cephalosporins. It also could be seen that cefotaxime sodium (**1**) and its degradation compounds could be separated well on the Click β-CD column, and this method can be used for the investigation of stability.

4. Conclusions

A HILIC method for the separation of seven cephalosporins was developed in this study. Buffer concentration and pH greatly affect the retention, which are largely due to their structures and physicochemical properties, such as acidity or basicity, cation or anion. Mobile phase with 10 mM ammonium formate at pH 6.8 was appropriate for most of cephalosporins. Different separation selectivity can be obtained by changing hydrophilic separation material, adjusting pH or adopting RP-LC mode. And the HILIC mode proves useful for the analysis of degradation products of cephalosporins.

Acknowledgments

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