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## Effect of eluent composition on retention behavior of anions in ion chromatography on anion-exchangers modified with heparin

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### Abstract

Effects of eluent composition on retention behavior of inorganic anions have been investigated in ion chromatography using anion-exchangers modified with heparin. Both cation and anion of the eluent affected the retention of analyte anions and unusual retention behavior was observed on the modified stationary phase. The retention time of anions decreased with decreasing eluent concentration when sodium sulfate, magnesium sulfate and chlorides of alkali metals were used as the eluent, whereas it increased with decreasing eluent concentration when aluminum sulfate, copper sulfate and sulfuric acid were used as the eluent. The retention of nitrate increased in the order of  $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Rb}^+$  and  $\text{Cs}^+$  when their chlorides were used as the eluent. When sodium perchlorate and chlorides of alkaline-earth metals were used as the eluent, the eluent should include heparin. Otherwise, the modifier was partially bled from the column. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Stationary phases, LC; Mobile phase composition; Retention behaviour; Inorganic anions

### 1. Introduction

In ion-exchange chromatography, retention and selectivity vary as the extent of the interaction between the eluent ion and the stationary phase. The main factors affecting this interaction, and ultimately the separation selectivity, include valency, size and structure of both eluent and analyte ions [1]. Eluents play a significant role in determining the overall selectivity of an ion-exchange separation.

In addition to the type of eluent, its concentration is one of the most important parameters affecting the retention. The importance for the selectivity of the eluent is its affinity toward the stationary phase. Eluent ions with a structure comparable to the stationary phase matrix, are characterized by a high affinity. At the same time, they also strongly affect the selectivity due to intense interactions with the stationary phase [2].

Anionic polysaccharides such as chondroitin sulfate and heparin have been successfully employed for the modification of anion-exchangers in ion chromatography, as reported previously [3–5]. The retention behavior of inorganic anions on these modified anion-exchangers depended on the mobile phase used. Unusual retention behavior was observed for silica-based strongly basic anion-exchangers

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modified with chondroitin sulfate C [3,4] or heparin [4,5]. The retention of anions decreased with decreasing eluent concentration when sodium sulfate or tartaric acid was used as the eluent, which allowed the use of tartaric acid as the eluent with a concentration as low as 30  $\mu\text{M}$ . The results showed a potential to improve the sensitivity [4]. Anion-exchangers modified with heparin retained both anions and cations, and simultaneous separation of anions and cations were demonstrated by using copper sulfate as the eluent [5]. However, it is not elucidated what type of eluents can be utilized for the columns modified with anionic polysaccharides.

Several papers reported that the effect of the eluent to study the retention mechanism in ion chromatography [6–12]. Mobile phases in ion-exchange chromatography are usually composed of diluted aqueous solution of acids, bases or salts. The composition of the mobile phase provides a great flexibility for manipulating an analyte retention in order to achieve the desired separation [13]. In this work, several kinds of eluents were used to observe the retention behavior of analyte anions, and then the retention mechanism will be explained from these results.

## 2. Experimental

### 2.1. Apparatus

A microcolumn liquid chromatograph was comprised from an MF-2 Microfeeder (Azumadenki Kogyo, Tokyo, Japan) equipped with an MS-GAN 050 gas-tight syringe (0.5 ml; Ito, Fuji, Japan), an ML-425 microvalve injector with an injection volume of 0.13  $\mu\text{l}$  (Jasco, Tokyo, Japan), a 100 $\times$ 0.32 mm I.D. microcolumn, and a UV-970 UV detector (Jasco, Tokyo, Japan). The flow-rate for the modification as well as for the separation was kept at 4.2  $\mu\text{l min}^{-1}$ . The UV detector was operated at 210 nm, and the data were acquired by a Chromatopac C-R4AX (Shimadzu, Kyoto, Japan). Size-exclusion chromatography of heparin was carried out by using TSKgel GMPW-XL columns (Tosoh, Tokyo, Japan; 300 $\times$ 7.8 mm I.D. $\times$ 2) and 0.2 M sodium nitrate as the eluent.

The microcolumns were prepared in the laboratory

from fused-silica tubing as reported [14]. The packing material employed was TSKgel IC-Anion-SW, a silica-based anion-exchanger with an ion-exchange capacity of 0.16 mequiv.  $\text{ml}^{-1}$ .

### 2.2. Reagents

The reagents employed were of guaranteed reagent grade and were obtained from Nacalai Tesque (Kyoto, Japan), unless otherwise noted. Purified water was prepared by using a Milli-Q Plus system (Millipore, Molsheim, France). Sodium salt of heparin was also obtained from Nacalai Tesque. The reagents were used without any further treatment. The eluents were prepared using the purified water.

### 2.3. Modification

The IC-Anion-SW column was first washed with water for 10 min and with 10 mM sodium sulfate for 2 h. Aqueous solution of 1.0% (w/v) heparin was then passed through the column for 2 h, followed by washing with water for ca. 30 min.

## 3. Results and discussion

### 3.1. Properties of heparin and anion-exchanger

Heparin is a mucopolysaccharide and it possesses carboxyl, sulfate and aminosulfonate groups as ionic moieties. Its weight-average molecular mass and polydispersity were determined by size-exclusion chromatography using TSKgel GMPW-XL columns and poly(ethyleneoxide) as the calibration standard. The weight-average molecular mass and polydispersity were  $2.2 \times 10^4$  and 1.24, respectively. TSKgel IC-Anion-SW employed in this work is a strongly basic anion-exchanger with an anion-exchange capacity of 0.16 mequiv.  $\text{ml}^{-1}$ , and its mean pore diameter is 14 nm.

### 3.2. Retention behavior of anions using various inorganic and organic eluents

Fig. 1 shows the retention behavior of nitrate on the modified stationary phase using several inorganic and organic eluents. It is seen that both anion and

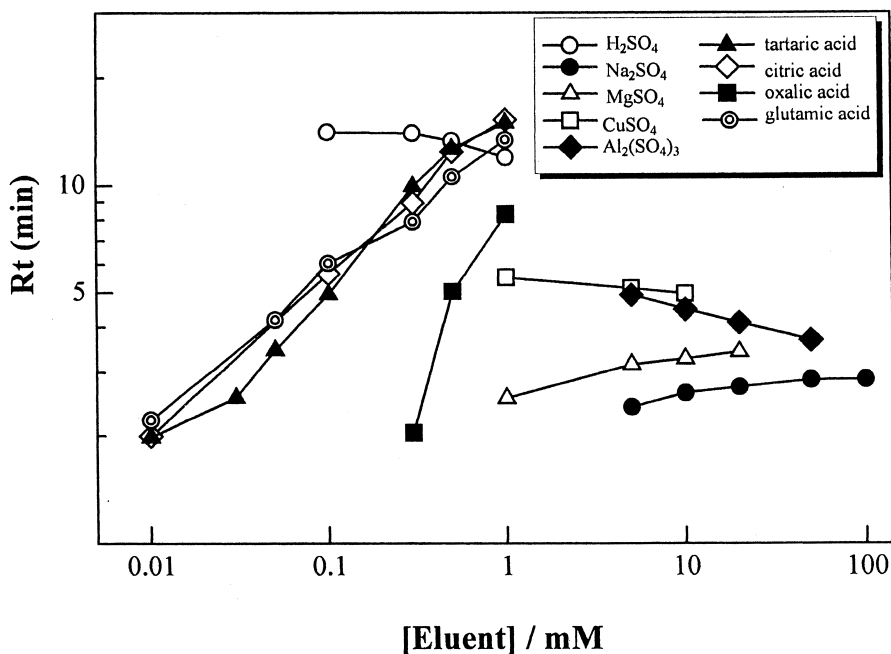


Fig. 1. Logarithm of retention time versus logarithm of the concentration of organic and inorganic eluents. Column, IC-Anion-SW (100×0.32 mm I.D.) modified with 1% heparin; eluent, sulfuric acid, sodium sulfate, magnesium sulfate, aluminum sulfate, copper sulfate, tartaric acid, oxalic acid, glutamic acid, and citric acid; flow-rate, 4.2  $\mu\text{l min}^{-1}$ ; injection volume, 0.13  $\mu\text{l}$ ; analyte, 0.1 mM sodium nitrate; wavelength used for UV detection, 210 nm.

cation of eluents affected the retention of analyte anions. Counter ions of the inorganic eluents showed different retention behavior of nitrate.  $\text{Mg}^{2+}$  and  $\text{Na}^+$  as the counter cation of sulfate eluents showed opposite retention behavior of the analyte anion to  $\text{Al}^{3+}$ ,  $\text{Cu}^{2+}$  and  $\text{H}^+$ . When inorganic eluents such as sodium sulfate and magnesium sulfate were used, the retention of the analyte anion decreased by decreasing the eluent concentration. Such an unusual retention behavior was also observed when organic eluents such as tartaric acid, oxalic acid, glutamic acid and citric acid were used. Decreasing the organic eluent concentration remarkably decreased the retention time of analyte anions. Nearly the same retention behavior was also observed for other test inorganic anions.

It is presumed that the anion-exchange sites exist underneath the cation-exchange sites, and the analyte anions should pass through the zone near the cation-exchange sites, where the analyte anions undergo repulsion. It is believed that the repulsion from the cation-exchange sites increases with decreasing

eluent concentration, leading to the decrease in the retention of analyte anions. On the contrary, the retention of analyte anions due to ion-exchange increases with decreasing eluent concentration. The net retention is determined by the above both contributions as well as by the retention of the counter cation, i.e., the eluent cation, on the cation-exchange sites. It is concluded that when the contribution of the effect of repulsion on the retention is dominant unusual retention behavior of anions on the modified stationary phase is observed. On the other hand, the eluent cations interact with the cation-exchange sites without undergoing repulsion from the anion-exchange sites. Owing to this situation, the retention behavior of cations is expected to be usual. In other words, the larger the retention of the counter cation, the larger the retention of the analyte anions. Unusual retention behavior is due to the presence of both anion-exchange and cation-exchange sites because analytes undergo both attraction and repulsion from the modified phase.

The cation-exchange site of the present stationary

phase is a quaternary ammonium group, whereas the anion-exchange sites are sulfate, aminosulfonate and carboxyl groups. The former is due to the residual anion-exchange sites of the substrate, whereas the latter is due to free anionic groups of the modifier.

Very dilute solution of tartaric acid could be used to separate inorganic anions. The separation of several anions (iodate, chloride, nitrite, bromide, nitrate, iodide and thiocyanate) could be achieved in 18 min using 0.4 mM tartaric acid as the eluent, as shown in Fig. 2. Although several reactions can occur, e.g., iodide and iodate, nitrite and iodide, thiocyanate and nitrite, these analyte anions were simultaneously separated to estimate the selectivity of the stationary phase in a single chromatographic run. Among the organic eluents used, tartaric acid was the most suitable for the separation of these anions at lower concentrations of eluent and in a shorter time. In addition, good separation of anions could not be achieved when glutamic acid was used as the eluent.

### 3.3. Retention behavior of anions using chlorides of alkali metals as the eluent

Chlorides of alkali metals could be successfully used as the eluent to separate inorganic anions on anion-exchangers modified with heparin. When chlorides of alkali metals were used as the eluent, unusual retention behavior was also observed as shown in Fig. 3; retention time of analyte anions decreased by decreasing the eluent concentration, except for cesium chloride. Unusual retention behavior is also due to the presence of both anion- and cation-exchange sites, as in the case of Fig. 1, and the nearly the same retention behavior is observed for other test analyte anions. The retention of nitrate increased in the order of LiCl, NaCl, KCl, RbCl and CsCl. The order is the same as the elution order of alkali metal ions in usual ion chromatography. The result supports that the present modified stationary phase retains both anions and cations. It is reasonable to conclude that anion-exchange of the analyte

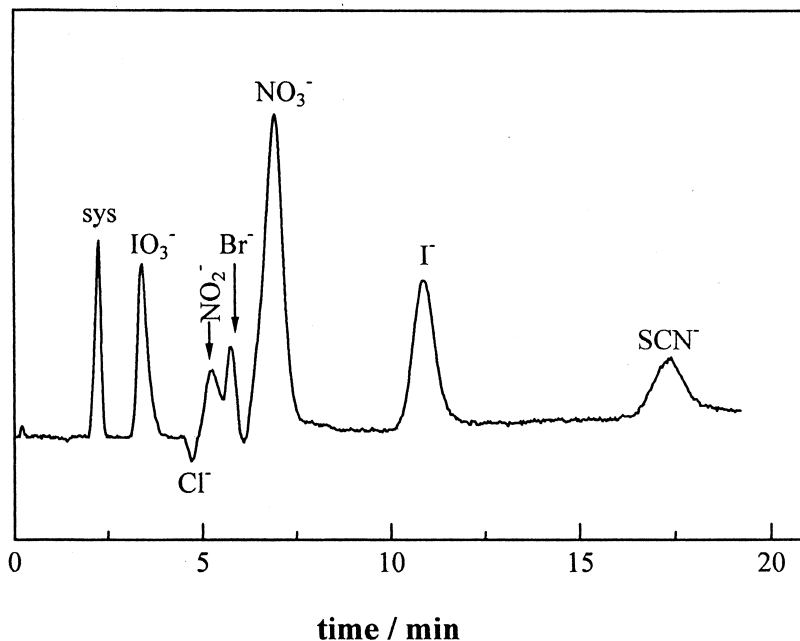


Fig. 2. Separation of anions on the modified anion-exchanger. Eluent, 0.4 mM tartaric acid; analytes, sodium iodate, sodium chloride, sodium nitrite, sodium bromide, sodium nitrate, sodium iodide and sodium thiocyanate, 0.1 mM each; other operating conditions as in Fig. 1.

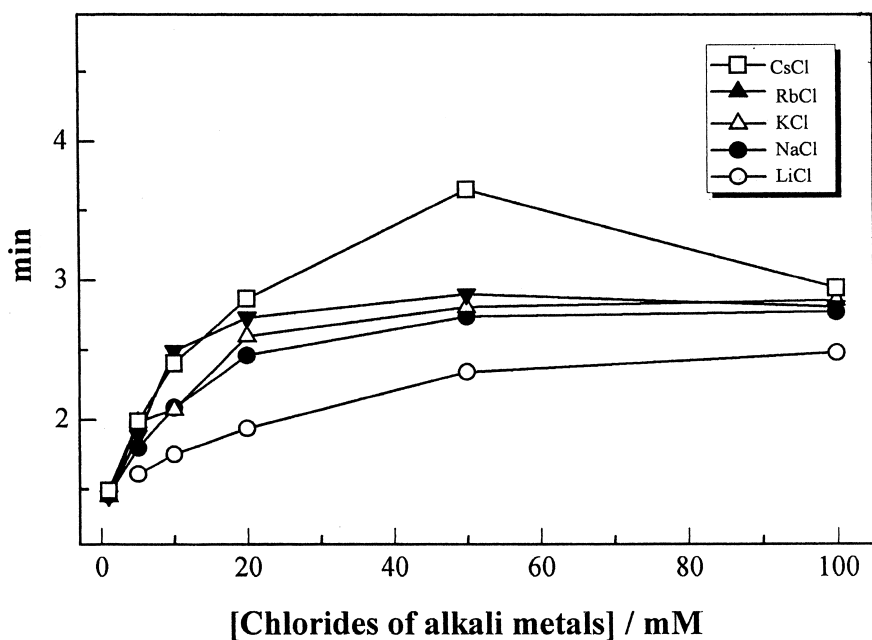


Fig. 3. Retention time (min) versus chlorides of alkali metals concentration. Eluents, cesium chloride, rubidium chloride, potassium chloride, sodium chloride, and lithium chloride; other operating conditions as in Fig. 1.

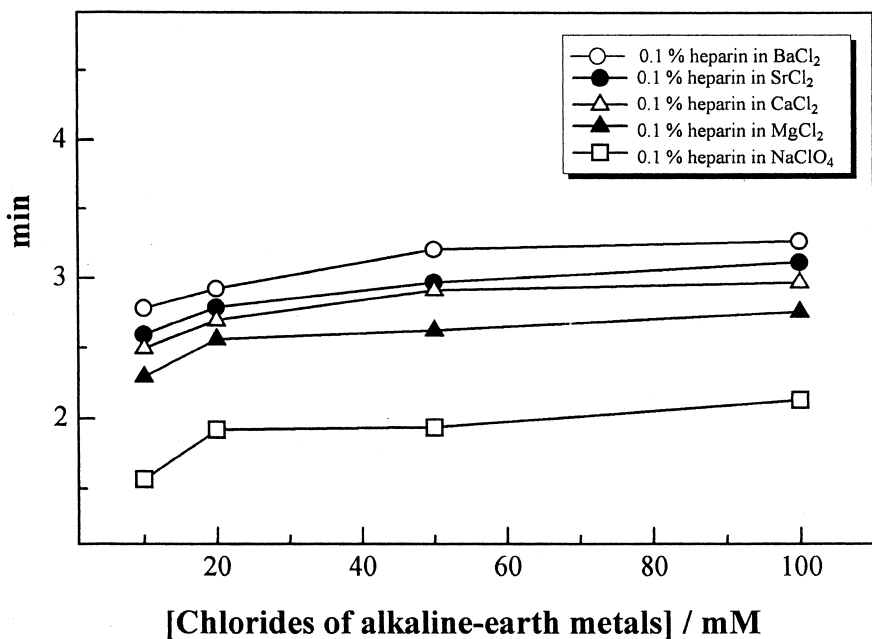


Fig. 4. Retention time (min) versus the concentration of chlorides of alkaline-earth metals and sodium perchlorate as the eluent containing 0.1% heparin. Eluents, 0.1% heparin in barium chloride, strontium chloride, calcium chloride, magnesium chloride and sodium perchlorate; other operating conditions as in Fig. 1.

anion as well as cation-exchange of the eluent cation, i.e., the counter ion of the analyte anion, contribute to the retention of the analyte anion. Eluent cation and anion as well as the eluent concentration therefore can affect the retention of anions. Since analyte anions move through the column together with eluent cations, it is presumed that the larger the interaction of the eluent cation with the stationary phase, the larger the overall retention of analyte anions. In addition, it is not certain why the retention time decreased at 100 mM for the CsCl eluent compared with that observed at 50 mM.

### 3.4. Retention behavior of anions using chlorides of alkaline-earth metals as the eluent

The retention of anions also increased in the order of  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$  when their chlorides were used as the eluent. However, the retention of anions increased by decreasing the concentration of the chlorides of alkaline-earth metals. It was found that the modifier was partially washed out of the

column when chlorides of alkaline-earth metals were used as the eluent. Therefore, the eluent should include the modifier to maintain the column condition. It was also found that sodium perchlorate eluted the modifier from the column. As shown in Fig. 4, when the eluent included 0.1% (w/v) heparin, the retention of nitrate decreased by decreasing the concentration of the eluent. The retention time of the analyte anion was remarkably decreased by using  $\text{BaCl}_2$ ,  $\text{SrCl}_2$ ,  $\text{CaCl}_2$ ,  $\text{MgCl}_2$  and  $\text{NaClO}_4$  containing 0.1% (w/v) heparin as the eluent for the separation of anions. The retention of analyte anions increased with increasing atomic number of the alkaline-earth metals. The elution order of the analyte coincides with that of alkaline-earth metals in common ion chromatography. Nearly the same retention patterns will be observed for other analyte anions.

Fig. 5 shows the separation of iodate, nitrate, iodide and thiocyanate on the stationary phase modified with heparin using 20 mM  $\text{BaCl}_2$  including 0.1% (w/v) heparin as the eluent (a) and on an unmodified stationary phase using 20 mM  $\text{BaCl}_2$  as

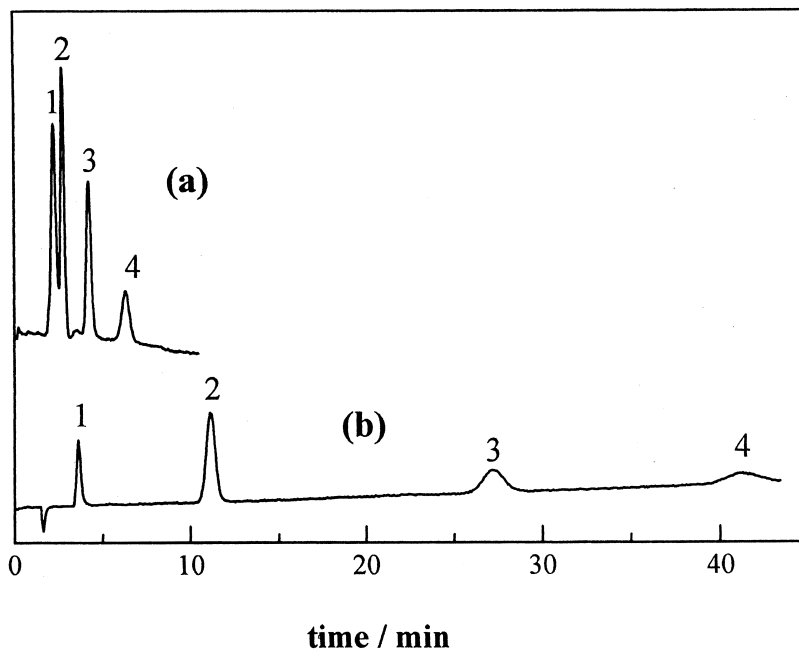


Fig. 5. Separation of anions on modified or unmodified anion-exchanger. Columns, IC-Anion-SW (100×0.32 mm I.D.) modified with 1% heparin (a) or unmodified (b); eluent, 20 mM barium chloride containing 0.1% heparin (a) or without heparin (b); flow-rate,  $4.2 \mu\text{l min}^{-1}$ ; analytes, 1=sodium iodate, 2=sodium nitrate, 3=sodium iodide and 4=sodium thiocyanate, 0.1 mM each; other operating conditions as in Fig. 1.

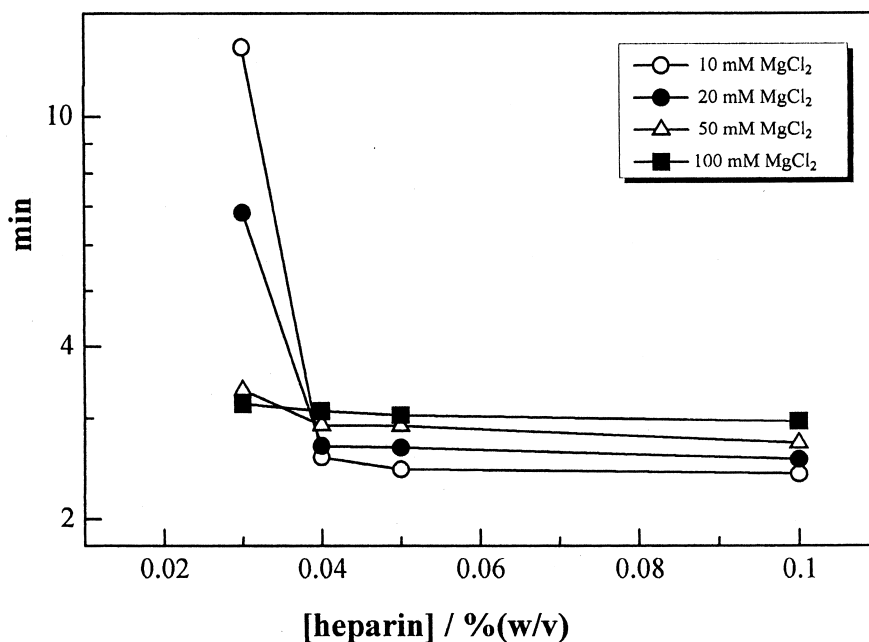


Fig. 6. Logarithm of retention time (min) versus the heparin concentration in the eluent Eluent, magnesium chloride containing heparin; other operating conditions as in Fig. 1.

the eluent (b). Chlorides of alkaline-earth metals including heparin could be successfully used as the eluent for the separation of inorganic anions in a shorter time.

The modifier contained in the mobile phase also affected the retention of analyte anions. Fig. 6 shows the retention of nitrate versus the concentration of heparin contained in the eluent. The retention of the analyte decreased with increasing heparin concentration. It is seen that the retention is nearly constant at the concentration higher than 0.04%. The result shows that the dynamic modification of the column with heparin can be maintained when the heparin concentration is higher than 0.04%. When the concentration of heparin was less than 0.04%, the retention of nitrate increased with decreasing eluent concentration. This means that at the concentrations less than 0.04% the dynamic modification is not complete and a portion of the modifier is being washed out of the column and usual retention behavior is observed. On the other hand, at the heparin concentrations higher than 0.04%, the larger the eluent concentration, the larger retention time was observed, as seen in Fig. 6.

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