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Investigation of the novel mixed-mode stationary phase for capillary electrochromatography I. Preparation and characterization of sulfonated naphthalimido-modified silyl silica gel

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Abstract

A novel packing material, 3-(4-sulfo-1,8-naphthalimido)propyl-modified silyl silica gel (SNAIP), was prepared for the use as a stationary phase of capillary electrochromatography (CEC). The sulfonic acid groups on SNAIP stationary phase contributed to the generation of electroosmotic flow (EOF) at low pH and served as a strong cation-exchanger. In CEC with SNAIP, a mixed-mode separation was predicted, comprising hydrophobic and electrostatic interactions as well as electrophoretic migration process. In order to understand the retention mechanism on SNAIP, effects of buffer pH, concentration, and mobile phase composition on EOF mobility and the retention factors of barbiturates and benzodiazepines were systematically investigated. Moreover, the retention behavior of barbiturates on SNAIP was investigated and compared with those on octadecyl silica (ODS), phenyl-bonded silica, and 3-(1,8-naphthalimido)propyl-modified silyl silica gel to confirm the presence of π–π interaction on its retention mechanism. It was observed that a column efficiency was more than 85,000 N/m for retained compounds and the relative standard deviations for the retention times of EOF marker, thiourea, and five barbiturates were below 2.5% (n = 4). Under an applied voltage of 20 kV and a mobile phase consisted of 5 mM phosphate (pH 3.8) and 40% methanol, the baseline separation of five barbiturates was achieved within 3 min.

Keywords: Stationary phases, electrochromatography; Hydrophobic interaction; Electrostatic interaction; π–π interaction; Electrochromatography; Barbiturates; Benzodiazepines; 3-(4-Sulfo-1,8-naphthalimido)propyl-modified silyl silica gel

1. Introduction

Capillary electrochromatography (CEC), which combines the features of capillary zone electrophoresis (CZE) and high performance liquid chromatography (HPLC), is a powerful separation technique with high efficiency, high resolution, and low consumption of mobile phase and sample [1–3]. After the first report on CEC in 1974 [4], the potential of CEC has been demonstrated by a burst of outstanding separations and applications [5–18].

The most commonly used stationary phase in CEC has been octadecyl silica (ODS), but in view of the need to maintain a high enough electroosmotic flow (EOF), it can only work in the mobile phase with relatively high pH. When mobile phases are used with low pH, the EOF decreases tremendously owing to the protonation of silanol groups on the surface of ODS. Most silica-based materials for reversed-phase (RP) HPLC have a low density of residual silanol groups and therefore are not suited for CEC. One way to overcome this problem is to use silica based strong cation exchanger (SCX) as a stationary phase [19–22] or mixed-mode stationary phase (C_{18}/SCX) [15,23–26]. The benefit of using this type phases in CEC is the contribution of a high EOF due to the ionized sulfonic acid group even

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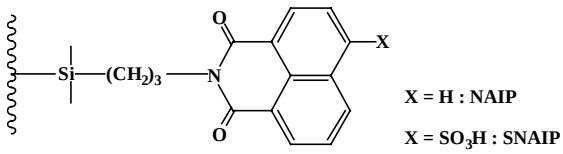


Fig. 1. Structures of NAIP and SNAIP.

at low pH. Rassi and co-workers [27,28] have developed a specially designed CEC stationary phase chemically bonding the sulfonic acid and octadecyl groups on silica and presented its application for nucleic acids.

In the initially described CEC, the major focus was on the separation behavior of neutral pharmaceuticals and aromatic hydrocarbons that were well studied in RP-HPLC. However, the most promising area for development of CEC now lies in the separation of charged analytes. Several attractive attempts to achieve a separation of charged analytes, especially of peptides and proteins, have been described [11,19,22–25,29–31]. Since charged analytes are hardly retained on the conventional RP stationary phases, most of these workers employed ion exchangers as a stationary phase or mixed-mode phase and found them a viable alternative. The mixed-mode phases meet the demands of separating many types of charged biomolecules with a selectivity different from that obtained in RP-HPLC and CZE. A better understanding of the separation mechanism on mixed-mode phases will enrich CEC of charged analytes.

Using a novel stationary phase, 3-(1,8-naphthalimido) propyl-modified silyl silica gel (NAIP, Fig. 1), we have recently proposed the HPLC and CEC separations of several biologically important substances that were based on hydrophobic and π–π interactions [32–34]. Also, its applicability for real sample analysis was fully demonstrated on the CEC of xanthine derivatives and barbiturates in rat brain microdialysate and human serum, respectively [35,36]. In this work, 3-(4-sulfo-1,8-naphthalimido)propyl-modified silyl silica gel (SNAIP, Fig. 1) was newly synthesized with the idea to use the fixed charge of sulfonic acid group as both the EOF generator and chromatographic retentive sites. The structure of SNAIP could be contributed to the retention and selectivity by three interactions including hydrophobic, electrostatic, and π–π interactions. Effects of operation parameters (e.g. buffer pH, concentration, and mobile phase composition) on EOF and the retention factors of barbiturates and benzodiazepines were systematically studied. This is the first attempt to employ the sulfonated naphthalimido-modified silica as a mixed-mode phase material for CEC separation.

2. Experimental

2.1. Instrumentation and materials

All the CEC experiments were performed on a CAPI-3200 system equipped with a photodiode array detector (Otsuka Electronics, Osaka, Japan). A Tosoh HPLC pump (Tokyo, Japan) was used to pack the materials into the capillary. Fused-silica capillaries (375 μm o.d. × 75 μm i.d.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). 4-Sulfo-1,8-naphthalic anhydride potassium salt was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, USA). Analytical grade of methanol and ethanol (Wako Pure Chemicals, Osaka) were used. The sources of barbiturates and benzodiazepines were as follows: barbital and phenobarbital from Fujisawa Astra Japan (Osaka), secobarbital sodium and triazolam from Yoshitomi Pharmaceutical Industries (Osaka), thiopental sodium from Tanabe Seiyaku (Osaka), nitrazepam and diazepam from Wako Pure Chemicals. The chemical structures, pK_a and log P values of tested barbiturates and benzodiazepines are summarized in Table 1.

2.2. Stationary phase synthesis

SNAIP was obtained by treating 3-aminopropyl silyl silica gel (2.5 g; particle size, 5 μm; pore diameter, 120 Å; Daiso, Osaka) with 4-sulfo-1,8-naphthalic anhydride (1 g, 2.89 mmol) in dimethylformamide (DMF, 40 ml) at 150 °C for 5 h in a sealed stainless steel vessel, followed by post-treatment (rinsing with DMF and methanol and then drying). The modification ratio of 3-aminopropyl siliyl silica gel with 4-sulfo-1,8-naphthalic anhydride was 0.549 mmol/g which was estimated from the percentage of carbon by elemental analysis.

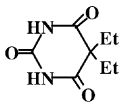
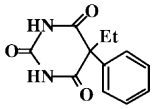
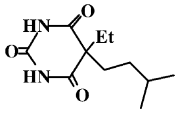
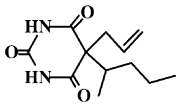
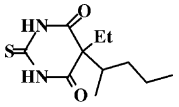
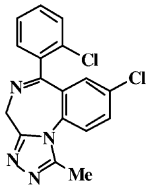
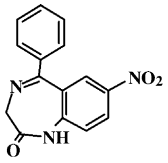
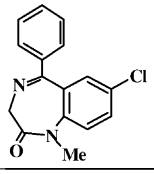
2.3. Column preparation

CEC columns packed with SNAIP were prepared by slurry packing technique as reported in our previous literatures [34–36]. After packing a slurry of SNAIP by the HPLC pump, the slurry solvent in the capillary was replaced with water and an outlet frit was made at 90 mm from inlet frit by sintering with a hot resistance wire. The polyimido coating was then burned away to make a detection window at 5 mm from outlet frit (i.e. 95 mm from inlet frit). All columns were 370 mm long with a packed length of 90 mm. The prepared capillary column was initially flushed with a mobile phase at 40 bar with the HPLC pump for 6 h. Then, elevated voltage from 1 to 15 kV was applied to the capillary column overnight for the equilibration.

2.4. Samples and mobile phases

All aqueous solutions were made with the water that was deionized and distilled by WG 220 (Yamato Scientific Co, Tokyo) and passed through a water purification system (Puric-Z, Organo Co, Tokyo). The stock solutions of the barbiturates and benzodiazepines were prepared by dissolving 1 mg of each compound in 1 ml of ethanol and methanol, respectively. The stock solutions were diluted to the desired concentration with a buffer prior to injection. Stock solutions of phosphate buffer were prepared by appropriate amount of

Table 1
Structures, log P and pK_a values of barbiturates and benzodiazepines as model compounds

	log P[a]	pK_a[a]	Structure
Barbiturates			
Barbital	0.68	7.95	
Phenobarbital	1.71	7.63	
Amobarbital	2.10	7.94	
Secobarbital	2.33	7.81	
Thiopental	3.00	7.76	
Benzodiazepines			
Triazolam	2.67	2.32	
Nitrazepam	2.84	3.19	
Diazepam	3.86	3.40	

[a] The values were taken from [40].

KH_2PO_4 in 100 ml water, then adjusting to desired pH by KOH or H_3PO_4. The phosphate buffer was filtered through a 0.45-μm membrane filter (Millipore Corporation, Bedford, MA, USA) before mixing with methanol and water for preparing mobile phase. The mobile phase was degassed thoroughly prior to use.

2.5. Electrochromatography

At the beginning of each day's work, the capillary column was conditioned with a mobile phase at 40 bar using the HPLC pump for 1 h and equilibrated by applying a low voltage of 1 kV for 30 min. The applied voltage ramped from 1 to 15 kV in 15 min and then held at 20 kV for 10 min for the equilibration. Instead of pressuring at both ends of the capillary column, the CEC system was thermostatically maintained at 18 °C throughout the analysis in order to avoid bubble formation within the capillary column. If not otherwise stated, the applied voltage in our experiments was 20 kV and the injections were made by applying a voltage of 15 kV for 6 s. The dual detection wavelength was set at 290 nm for thiopental and 210 nm for the others. The EOF mobility (μ_{EOF}) was calculated as follows:

$$\mu_{EOF} = \frac{u}{E}$$

where $E = 20\,kV/370\,mm$; $u = 95\,mm/t_0$.

In an attempt to describe the retention of charged analytes in CEC, Rathore and coworkers [37–39] have defined a CEC retention factor, k^*, as:

$$k^* = \frac{t_m(1 + k_e^*) - t_0}{t_0}$$

where t_m and t_0 denote the retention time of the analyte and that of an inert and neutral tracer (EOF marker), respectively. Thiourea was chosen as an EOF marker in this study. k_e^* is the velocity factor, indicating the contribution of electrophoretic mobility to the separation of charged analytes in CEC, and is given by:

$$k_e^* = \frac{\mu_p}{\mu_0}$$

where μ_p is the electrophoretic mobility of the analyte which is obtained from CZE measurements under the same conditions as the CEC separation. The interstitial EOF mobility in the CEC column, μ_0, is equal to the apparent EOF mobility within the CEC column multiplied by the ratio of current in open tube to that in packed column [37–39]. For neutral analytes, $k_e^* = 0$, consequently the k^* is equal to k', retention factor as defined in HPLC. The column efficiency N was calculated from the number of theoretical plates per meter:

$$N = 5.55\left(\frac{t_r}{w_{0.5}}\right)^2$$

where $w_{0.5}$ is peak width at half height.

3. Results and discussion

3.1. Column performance

The influence of buffer pH on the EOF mobility (μ_{EOF}) was studied in the pH range of 2.5–7.5 using the mobile phase composed of 5 mM phosphate buffer and 40% methanol (Fig. 2). By using SNAIP column, μ_{EOF} became 1.19 and $1.55 \times 10^{-8}\,m^2\,V^{-1}\,s^{-1}$ at pH 2.5 and 3.0, respectively. These corresponded to 1.29 and 1.43-fold increase of μ_{EOF} when compared with NAIP column using lower

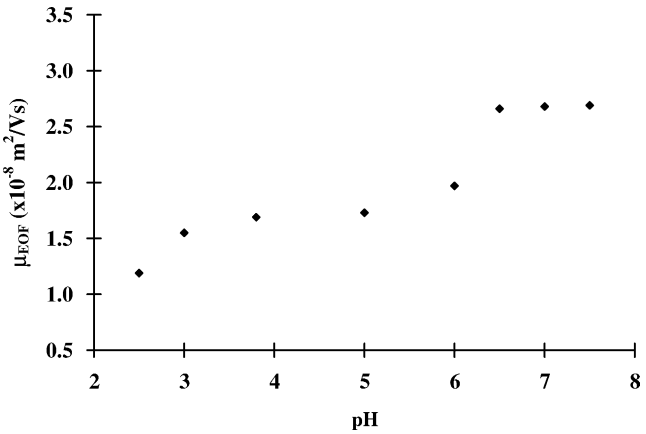


Fig. 2. Dependence of EOF mobility with SNAIP on the pH of mobile phase. *Conditions*: column, total length 370 mm (packed length 90 mm) × 75 μm i.d. × 375 μm o.d.; mobile phase, 5 mM phosphate–methanol (60:40, v/v) at different pH values; applied voltage, 20 kV; temperature, 18 °C.

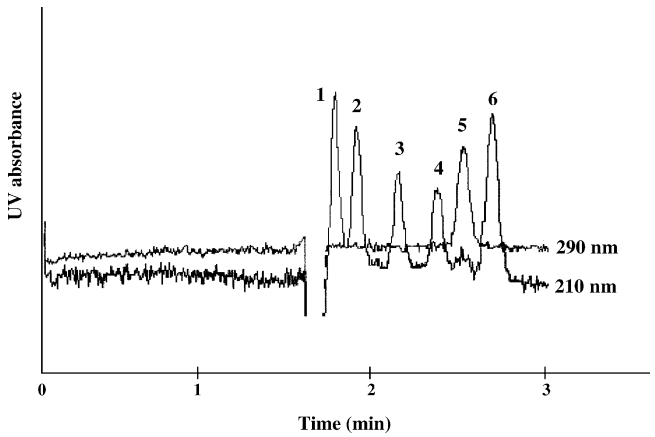


Fig. 4. Electrochromatogram of barbiturates with SNAIP. Mobile phase: 5 mM phosphate (pH 3.8)–methanol (60:40, v/v). Other conditions as in Fig. 2. Peaks: (1) thiourea; (2) barbital; (3) amobarbital; (4) secobarbital; (5) thiopental; (6) phenobarbital.

phosphate concentration (1 mM) [34] which generally provides faster EOF in CEC. This increase was attributed to the introduction of sulfonic acid groups, which was imperative for a fast separation at low pH. The slight increase from pH 3.0 to 6.0 and steep increase from pH 6.0 to 6.5 might be due to the rising of silanol ionization. The surface of the capillary wall and SNAIP was completely ionized in the pH range of 6.5–7.5.

Effect of methanol content (30–60%) in the mobile phase on μ_{EOF} was also investigated by keeping the phosphate concentration at 5 mM and the pH of 3.8 (Fig. 3). An increase of μ_{EOF} with a decrease in methanol content was obtained, which was similar to previous works [41–43]. This tendency provides a significant advantage of suppressing the longer separation time with lowering methanol content that is generally used for better resolution in RP chromatographic separation. As shown in Fig. 4, a rapid baseline separation of five barbiturates was achieved within 3 min by an applied voltage of 20 kV and a mobile phase of 40% methanol in 5 mM phosphate (pH 3.8).

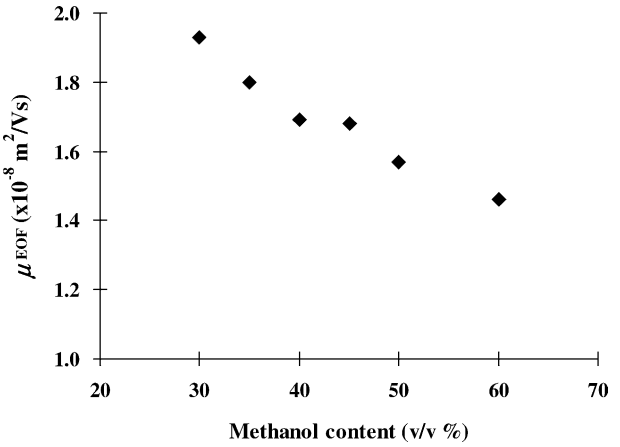


Fig. 3. Dependence of EOF mobility with SNAIP on the content of methanol in mobile phase. Mobile phase: 5 mM phosphate (pH 3.8)–methanol with varying methanol content. Other conditions as in Fig. 2.

With SNAIP column, column efficiency of thiopental and phenobarbital was 91,000 and 85,000 N/m, respectively. The good reproducibility was demonstrated with the relative standard deviations ($\leqq$2.5%, $n = 4$) for the retention times of thiourea and five barbiturates. An SNAIP column could be used for more than 6 months without distinct loss of resolution and efficiency. In addition, no current breakdown or formation of bubbles in SNAIP column was observed, even though the current reached 40 μA.

3.2. Separation of benzodiazepines

The prepared SNAIP column was applied for the separation of benzodiazepines at low pH and the separation mechanism of the basic compounds on SNAIP column was studied. The chromatographic part of the separation mechanism in CEC with SNAIP is expected to involve electrostatic and/or hydrophobic interactions that can be regulated by the ionic strength and/or the content of organic modifier in mobile phase, respectively.

The effect of phosphate concentration on the retention of benzodiazepines in the range from 5 to 40 mM was investigated with the mobile phase of phosphate buffer (pH 3.8) and 50% methanol (Fig. 5). It was seen that the retention of each benzodiazepine decreased with increasing the concentration. The decrease in the retention was presumably a result of weakening electrostatic binding of positively charged benzodiazepines to the negatively charged sulfonic acid groups. This explanation was supported by a linear relationship between the logarithmic values of k^* and phosphate concentrations, which is a typical behavior in ion-exchange chromatography [19,44]. In addition, increasing the methanol content in the mobile phase resulted in a decrease in the retention of benzodiazepines (data not shown).

In order to distinguish the contribution of electrostatic and

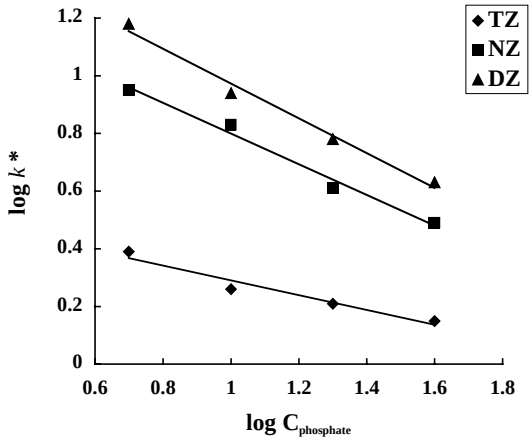


Fig. 5. Influence of the phosphate concentration with SNAIP on the retention factors of benzodiazepines. Double logarithmic plots of retention factors against the phosphate concentration. Mobile phase: phosphate (pH 3.8)–methanol (50:50, v/v); electrokinetic injection, 15 kV × 10 s. Other conditions as in Fig. 2.

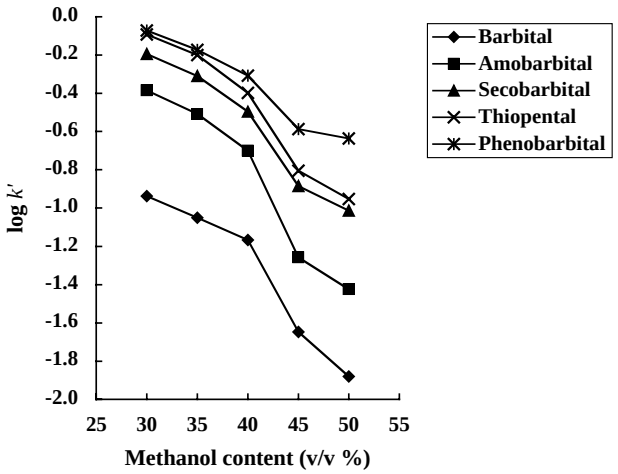


Fig. 6. Influence of methanol content with SNAIP on the retention factors of barbiturates. Mobile phase: 5 mM phosphate (pH 3.8)–methanol with varying methanol content. Other conditions as in Fig. 2.

hydrophobic interactions from the electrophoretic migration for the separation of benzodiazepines, the CZE experiment was also carried out under the same condition as CEC. The obtained migration times for benzodiazepines in CZE and the corresponding retention times in CEC with SNAIP are summarized in Table 2. In CZE at pH 2.5, it can be seen that diazepam and nitrazepam migrated before the EOF marker, thiourea, whilst triazolam eluting near to thiourea. From these results, it was concluded that the elution process of basic compounds in CEC with SNAIP was mediated by a combination of both electrophoretic migration process and retention mechanism including hydrophobic as well as electrostatic interactions.

3.3. Separation of barbiturates

As expected in LC, the retention of barbiturates decreased with an increase in methanol content, which meant the existence of the hydrophobic interaction between analytes and SNAIP. The $\log k'$ values of five barbiturates were plotted against the content of methanol using 5 mM phosphate at pH 3.8 (Fig. 6). A linear relationship between $\log k'$ and methanol content was not obtained and the two intervals showing different slopes were observed. In the first interval (30–40%), $\log k'$ versus methanol content decreased slower than in the range of the content varied from 40 to 50%. This phenomenon contradicted previous works concerning the separation of aromatic hydrocarbons [45,46]. Theoretically, $\log k'$ versus methanol content plots are directly related to the adsorption isotherm of methanol that increases as the content of methanol in mobile phase decreases. The range where $\log k'$ versus methanol content of analyte decreases more steeply should be related to the interval where the isotherm of methanol increases rapidly. However, these differences have yet to be well resolved.

On the other hand, in CEC with ODS (Hypersil C_{18}), Euerby et al. [12] demonstrated that the elution order of studied barbiturates was completely consistent with their hydrophobicity and phenobarbital eluted before secobarbital even using a phenyl-bonded stationary phase with the retention by $\pi-\pi$ interaction. In CEC with NAIP [34,36], like ODS, the elution order of four barbiturates (barbital, phenobarbital, secobarbital, and thiopental) was according to their hydrophobicity (Fig. 7a). Marked difference in selectivity was apparent between SNAIP and these stationary phases. As shown in Fig. 7b, a different elution order was observed only for phenobarbital possessing an aromatic moiety that eluted last in spite of its lower $\log P$ value, while the order of the others was according to their $\log P$ values. In order to confirm the presence of $\pi-\pi$ interaction, plots of $\log k'$ against $\log P$ values for tested barbiturates were drawn (Fig. 8). A linear relationship was obtained for tested barbiturates, except for phenobarbital. From these results, the difference in selectivity came from $\pi-\pi$ interaction between SNAIP and the aromatic moiety of phenobarbital. Naphthalyl group modified with electron-acceptor group (i.e. sulfonic acid group) became π-acidic and thus the impact of the $\pi-\pi$ interaction could be pronounced.

Table 2
Comparison between CZE and CEC for elution times of benzodiazepines[a]

	Elution time (min)	
	CZE	CEC
Diazepam	2.68	–[b]
Nitrazepam	2.81	120.92
Triazolam	3.03	19.37
Thiourea (EOF marker)	3.01	2.89

[a] *Conditions*: mobile phase, 5 mM phosphate (pH 2.5)–methanol (50:50 v/v); voltage, 20 kV.
[b] Not eluted.

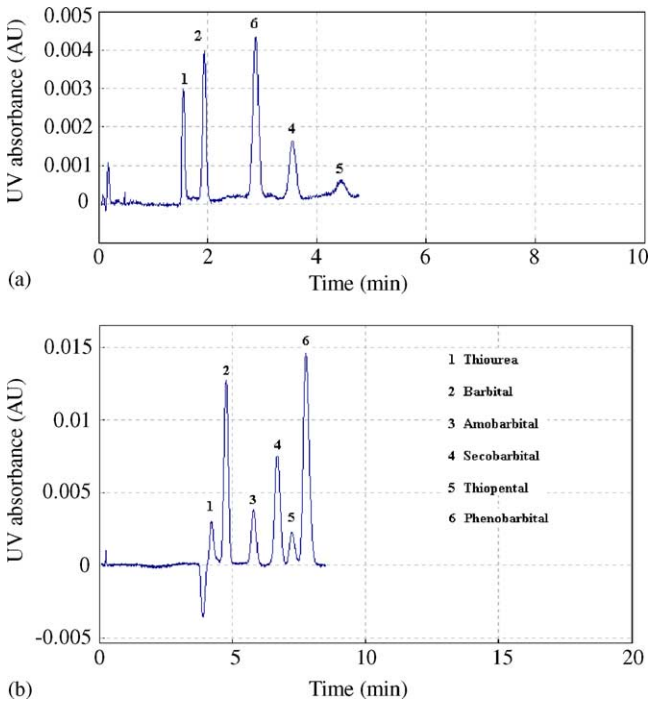


Fig. 7. Electrochromatographic separations of barbiturates with NAIP and SNAIP. (a) Stationary phase, NAIP; mobile phase, 1 mM citrate (pH 5.0)–methanol (60:40, v/v); applied voltage, 20 kV. (b) Stationary phase, SNAIP; mobile phase, 5 mM phosphate (pH 3.8)–methanol (60:40, v/v); applied voltage, 10 kV.

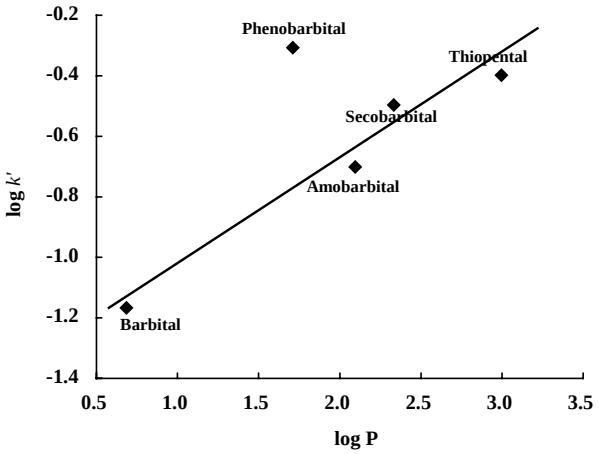


Fig. 8. Relationship between log k' and log P with SNAIP for barbiturates. Mobile phase: 5 mM phosphate (pH 3.8)–methanol (60:40, v/v). Other conditions as in Fig. 2.

4. Conclusions

The experimental data have confirmed that the separation mechanism in CEC with SNAIP stationary phase was a hybrid of electrophoretic migration and chromatographic retention involving hydrophobic, electrostatic as well as π–π interactions. Using low pH mobile phase, the baseline separation of five barbiturates in less than 3 min was observed due to the higher EOF. SNAIP is easily prepared on a single reaction and shows a different selectivity than conventional RP stationary phases. The application of the SNAIP stationary phase for the CEC of a wide range of charged species, such as amino acids and peptides, will be the topic of upcoming publications.

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