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Chromatographic evaluation of reversed-phase/anion-exchange/cation-exchange trimodal stationary phases prepared by electrostatically driven self-assembly process

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ABSTRACT

This work describes chromatographic properties of reversed-phase/cation-exchange/anion-exchange trimodal stationary phases. These stationary phases were based on high-purity porous spherical silica particles coated with nano-polymer beads using an electrostatically driven self-assembly process. The inner-pore area of the material was modified covalently with an organic layer that provided both reversed-phase and anion-exchange properties while the outer surface was coated with nano-sized polymer beads with strong cation-exchange characteristics. This design ensured spatial separation of the anion-exchange and the cation-exchange regions, and allowed reversed-phase, anion-exchange and cation-exchange retention mechanisms to function simultaneously. Chromatographic evaluation of ions and small molecules suggested that retention of ionic analytes was influenced by the ionic strength, pH, and mobile phase organic solvent content, and governed by both ion-exchange and hydrophobic interactions. Meanwhile, neutral analytes were retained by hydrophobic interaction and was mainly affected by mobile phase organic solvent content. Depending on the specific application, selectivity could be optimized by adjusting the anion-exchange/cation-exchange capacity ratio (selectivity), which was achieved experimentally by using porous silica particles with different surface areas.

1. Introduction

Because of the complexity and variety of analytes in terms of hydrophobicity and charge in HPLC applications, it is highly challenging to separate acidic, basic, and neutral molecules using any single separation mode. Reversed-phase liquid chromatography (RPLC) is the most commonly used separation mode for a broad range of analytes [1], but it often fails to retain highly hydrophilic analytes, such as catecholamines and inorganic ions (e.g., Na^+ and Cl^-). Although ion-pairing liquid chromatography improves the retention and selectivity of hydrophilic ionizable analytes, it requires long equilibration times, a dedicated column, and usually a MS-incompatible mobile phase [1,2]. Ion chromatography (IC) provides a reliable, selective, and sensitive solution to the analysis of ionic analytes [3,4], but this technique is not suitable for neutral analyte determinations. Hydrophilic Interaction Chromatography (HILIC) complements the aforementioned techniques and is suitable for analyzing highly hydrophilic analytes [5,6]. However, challenges include solubility of the analytes in the mobile phase and greater organic solvent consumption compared to other separation modes.

Mixed-mode chromatography combines both reversed-phase (RP) and ion-exchange (IEX) retention mechanisms. Compared to RP, mixed-mode chromatography offers adequate retention of charged analytes, selectivity complementary to RP, IEX, and HILIC columns, and adjustable selectivity, thus providing greater flexibility in method development [7,8]. Because of the complexity and variety of analytes in samples submitted for HPLC, it is advantageous to separate acidic, basic, and neutral molecules in a single analysis. This necessitates a stationary phase that provides anion-exchange, cation-exchange, and reversed-phase interactions at the same time. Several trimodal columns of different distinctive chemistry designs have been reported. One trimodal phase consists of uniformly blended packing material with two types of porous silica particles: silica modified with C18 and cation-exchange silyl ligands and silica modified with C18 and anion-exchange silyl ligands [9]. However, the resulting has an inherent drawback – undesirable selectivity drift due to the difference in hydrolytic stability between the RP ligand and the IEX ligand bonding sites [10]. Another trimodal phase is based on silica particles bonded with a silane containing reversed-phase, cationic, and anionic functional groups on the same ligand. The cationic group is close to

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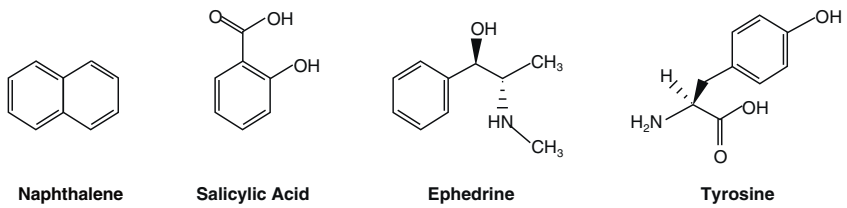

Fig. 1. Structures of test probes in this study.

the silica surface and is separated from anionic group by a long hydrophobic alkyl chain [11]. Despite the fact that this material has both anionic and cationic functional groups, they can neutralize one another due to their proximity promoted by the flexible alkyl linker, if both functional groups are ionized. Consequently, the phase behaves as a salt-exchange material rather than an ion-exchanger in which both anion-exchange and cation-exchange mechanisms function without mutual interferences. The third trimodal phase, the subject of this work, is prepared from an electrostatically driven self-assembly process. Its chemistry design creates a spatial separation of the anion-exchange and cation-exchange regions, and allows reversed-phase, cation-exchange, and anion-exchange retention mechanisms to function simultaneously and the resulting selectivity can be controlled with greater flexibility.

The objective of this work was to investigate the retention mechanism and chromatographic properties of the aforementioned trimodal stationary phases prepared from the electrostatically driven self-assembly process. The retention dependency of mobile phase ionic strength, pH, organic solvent, and electrolyte type were studied. Some separations are also developed to demonstrate the application of these unique packing materials.

2. Experimental

2.1. Separation columns

Stationary phases were prepared from high-purity spherical silica particles. Phase **A** used silica gel with the following physical properties: average particle size, 5-μm; specific surface area, 300 m^2/g; mean pore size, 120 Å; specific pore volume, 1.0 mL/g. Phase **B** used silica gel with the following physical properties: average particle size, 3-μm; specific surface area, 100 m^2/g; mean pore size, 300 Å; specific pore volume, 1.0 mL/g. Both phases were prepared using the same surface modification procedure. Columns were packed in 4.6 × 50-mm, 3.0 × 50-mm or 3.0 × 100-mm dimensions using Type 316 stainless steel tubing and a conventional slurry packing method [1]. The column packed with phase **B** was a commercial product under the name of Acclaim Trinity P1 (Dionex, Sunnyvale, CA, USA).

2.2. Reagents and materials

HPLC grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, USA). De-ionized water (>18 MΩ) was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Ammonium acetate salt (99.99+% pure) other reagents, and all standards were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Instrumentation

Separations were performed on a modular UltiMate™ 3000 HPLC System (Dionex) equipped with a LPG 3600 gradient pump, WPS-3000 Autosampler, TCC-3200 column oven, and PDA-3000 detector. For alkyl quaternary amines and pharmaceutical counterions detection, a Corona *ultra* detector (ESA – a Dionex Company, Chelmsford, MA, USA) or a Sedex-85 evaporative light scattering detector (Sedere, Alfortville, France) was used. Chromeleon® 6.8 Chromatography Management Software (Dionex) was used for system control and data processing.

2.4. Chromatographic conditions

Naphthalene (neutral), ephedrine (basic, p*K*a = 9.6), salicylic acid (acidic, p*K*a = 2.97), and tyrosine (zwitterionic, pKa_1 = 2.20, pKa_2 = 9.11, pKa_3 = 10.07, and isoelectric point = 5.66) were used to study the retention behavior. Their structures are illustrated in Fig. 1.

In the retention-ionic strength dependency study, mobile phases were generated by proportioning from four components: A – acetonitrile; B – 0.85% phosphoric acid; C – 100 mmol L^{-1} NaCl; D – D.I. water. While acetonitrile and 0.85% phosphoric acid were kept constant at 40% (v/v) and 10% (v/v), respectively, the ionic strength of mobile phase was controlled by various ratios of NaCl solution to D.I. water. The retention factors were recorded at 5, 10, 20, 30, and 40 mmol L^{-1} NaCl.

In the retention-salt dependency study, mobile phases were generated by proportioning from four components: A – acetonitrile; B – 0.85% phosphoric acid; C – 100 mmol L^{-1} salt; D – D.I. water. While acetonitrile and 0.85% phosphoric acid were kept constant at 40% (v/v) and 10% (v/v), respectively, the ionic strength of mobile phase was controlled by various ratios of the salt solution to D.I. water. NaCl and $NaClO_4$ were used to compare the anion effect. LiCl, NaCl and KCl were used to study the cation effect. The retention factors were recorded at 5, 10, 20, 30 and 40 mmol L^{-1} salt contents. The retention-ionic strength curves of different salts were compared.

In the retention-pH dependency study, mobile phases were generated by proportioning from three components: A – acetonitrile; B – 100 mmol L^{-1} phosphate buffer (pH 2.3 or 6.5); C – D.I. water. Buffer concentrations were all normalized to Na^+. While acetonitrile was kept constant at 40% (v/v), buffer concentrations were controlled by various ratios of phosphate buffer to D.I. water. The retention factors were recorded at 10, 20, 30, and 40 mmol L^{-1} Na^+ concentration. The retention-buffer concentration curves for both pH 2.3 and pH 6.5 phosphate buffers were compared.

In the retention-organic solvent dependency study, mobile phases were generated by proportioning from four components: A – acetonitrile; B – 0.85% phosphoric acid; C – 100 mmol L^{-1} NaCl; D – D.I. water. While both 0.85% phosphoric acid and NaCl solutions were kept constant at 10% (v/v), the contents of acetonitrile in the mobile phase were controlled by various ratios of acetonitrile to D.I. water. The retention factors were recorded at 30, 40, 50, 60, 70 and 80% (v/v) acetonitrile.

The void time t_0 was determined by examining the disturbance in UV spectrum as a PDA detector was used. The retention time (k') was defined as $(t_R - t_0)/t_0$. The detailed chromatographic conditions can be found in figure captions.

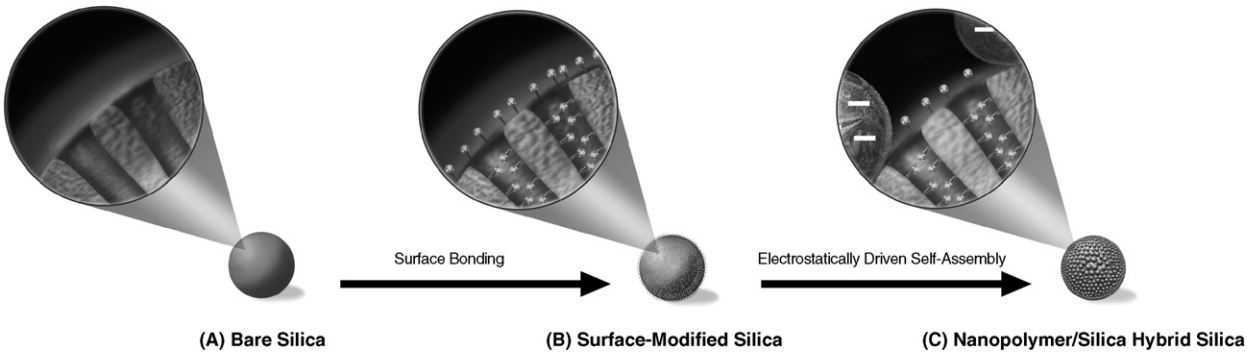

Fig. 2. Synthetic route of forming nanopolymer/silica hybrid material using an electrostatically driven self-assembly process. (A) Bare silica; (B) surface-modified silica; (C) nanopolymer/silica hybrid silica.

3. Results and discussion

3.1. Phase design and preparation

Organic/inorganic (O/I) hybrid particles have great potential for a variety of applications [12]. O/I particles can be constructed by assembling preformed organic and inorganic components in the form of particles or macromolecules. The properties of such materials depend not only on the chemical nature of the organic and inorganic components but also on the spatial arrangement of the different phases. It is known that complex ordered architectures can form spontaneously by self-assembly [13]. The interaction between particles of different characteristics (i.e., sizes, charges, and chemical composition) is known as heterocoagulation. Attractions between positively charged and negatively charged particles provide a viable approach for the formation of raspberry-like O/I particles made of an inorganic core surrounded by smaller polymer particles via an electrostatically driven self-assembly process [14].

Many HPLC stationary phases are based on porous spherical silica particles with surface area and pore size ranging from 50 to 500 m^2/g and from 60 to 300 Å, respectively. Due to the porous nature of silica particles, the inner-pore area contributes to most of the surface area. When the silica surface, including both inner-pore area and outer-pore surface, is covalently modified by silyl ligands bearing one type of charge, nano-sized polymer beads that are larger than the pore size and carry the opposite charge can be irreversibly attached to the outer-surface through electrostatic attraction while excluded from entering the inner-pore area because of steric hindrance. The resulting material has one charge in the inner-pore area and the opposite charge on the outer surface. Because of the spatial separation of positively charged and negatively charged domains, this type of materials can function as both anion-exchanger and cation-exchanger concurrently without mutual interferences.

Fig. 2 illustrates a two-step synthetic route for such O/I hybrid material using electrostatically driven self-assembly. First, porous silica particles are covalently modified with silyl ligands containing both hydrophobic alkyl chain (for hydrophobic retention) and terminus tertiary amine (for weak anion-exchange retention) to obtain surface modified silica particles. Secondly, nano-sized fully sulfonated polystyrene-divinylbenzene polymer beads are allowed to mix with above modified silica particles under proper conditions such that both components are ionized, to ensure an electro-statically driven self-assembly process. Because the size of nano-polymer beads is in the range of 600–3000 Å, much larger than the pore size of silica substrate which pore size is 120 or 300 Å, these charged nano-sized polymer beads are selectively and permanently attached to the outer surface area by electrostatic attraction, but are excluded from the inner-pore area due to steric hindrance The resulting material possesses reversed-phase, weak anion-exchange and strong cation-exchange properties. During phase design, desirable selectivity can be obtained by choosing appropriate surface area and pore size of silica particles, size and charge density of nano-polymer beads, and conditions for the self-assembly process. Because the making of such materials is outside the scope of this study, we will focus on chromatographic evaluation in the reminder of this paper.

3.2. Chromatographic evaluation

3.2.1. Salt concentration effect

The retention of charged analytes is heavily affected by mobile phase ionic strength. Both ephedrine (cationic) and salicylic acid (anionic) are charged under the test conditions. As shown in Fig. 3, retention of both ephedrine and salicylic acid decrease with the increase of NaCl in the mobile phase. In other words, retentions of ephedrine and salicylic acid decrease with the increase of their corresponding competing ions – Na^+ and Cl^-, respectively. These observations are typical characteristics of cation-exchange and

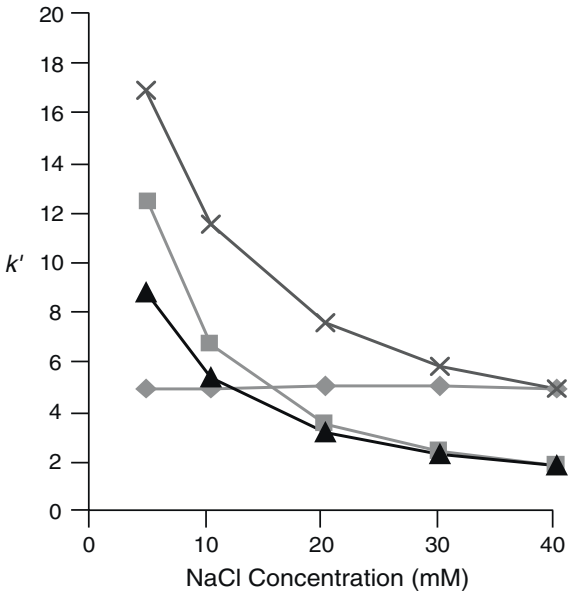

Fig. 3. Effect of salt concentration. Column, trimode phase **A**, 4.6 × 50-mm format; mobile phase, acetonitrile/1% H_3PO_4/5–40 mmol L^{-1} (total concentration) NaCl aqueous solution (40:10:50, v/v/v); flow rate, 1.0 mL min^{-1}; injection volume, 3 μL; temperature, 30 °C; and detection, UV at 210 nm. Analytes (0.2 mg mL^{-1} each): naphthalene (diamond), salicylic acid (cross), ephedrine (square), tyrosine (triangle).

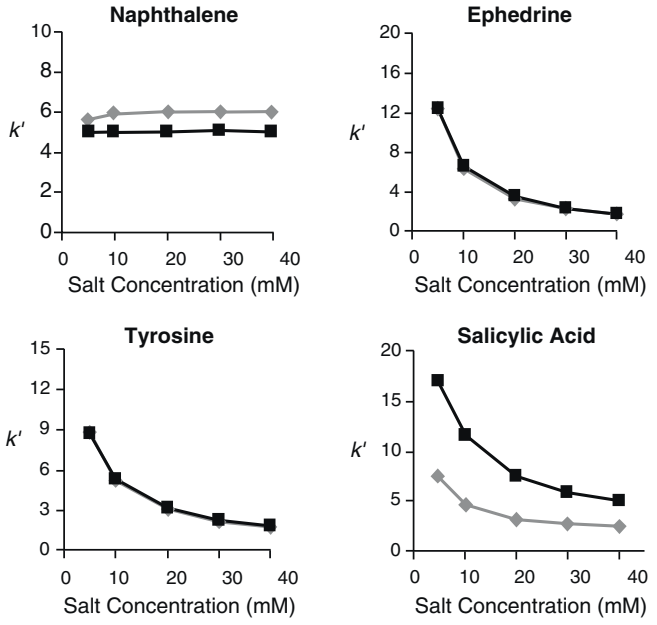

Fig. 4. Effect of anion type. Column, trimode phase **A**, 4.6 × 50-mm format; mobile phase, acetonitrile/1% H_3PO_4/5–40 mmol L^{-1} (total concentration) sodium salt aqueous solution (40:10:50, v/v/v); flow rate, 1.0 mL min^{-1}; injection volume, 3 μL; temperature, 30 °C; and detection, UV at 210 nm. Salt additive: NaCl (square), $NaClO_4$ (diamond).

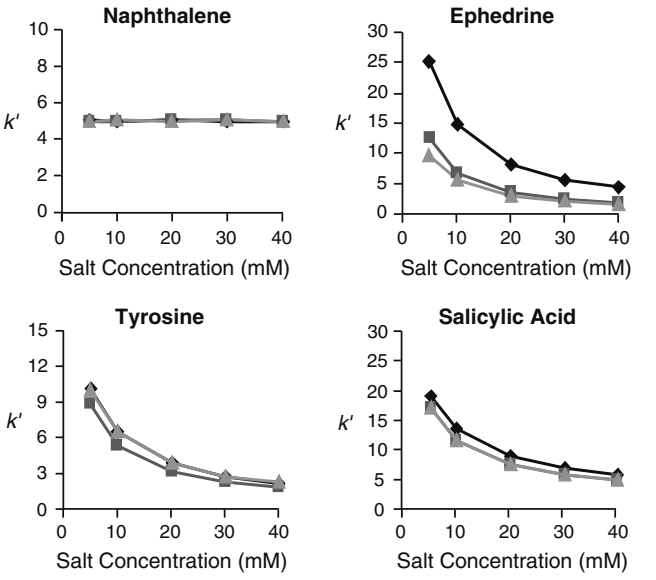

Fig. 5. Effect of cation type. Column, trimode phase **A**, 4.6 × 50-mm format; mobile phase, acetonitrile/1% H_3PO_4/5–40 mmol L^{-1} (total concentration) chloride salt aqueous solution (40:10:50, v/v/v); flow rate, 1.0 mL min^{-1}; injection volume, 3 μL; temperature, 30 °C; and detection, UV at 210 nm. Salt additive: LiCl (diamond), NaCl (square), KCl (triangle).

anion-exchange processes. Although tyrosine is zwitterionic by nature, it has net positive charge at pH 2.3 as its isoelectric point is 5.66, thus it behaves as a cation. On the other hand, ionic strength exhibits no effect on neutral analytes: retention of naphthalene did not change with NaCl concentration.

3.2.2. Salt type effect

The type of salt additive in the mobile phase also has effect on analyte retention. We compared NaCl and $NaClO_4$ for anion effect, in which case competing cations were the same but competing anions were different – Cl^- vs. ClO_4^-. Because ClO_4^- is a stronger competing anion than Cl^-, at the same solvent concentration salicylic acid exhibited lower retention in the $NaClO_4$ containing mobile phase than the NaCl containing one (Fig. 4). Conversely, ephedrine and tyrosine (both cationic under testing conditions) were not affected by different anions, and thus exhibited overlapping retention-salt concentration curves. Naphthalene (neutral) exhibited somewhat higher retention in the presence of $NaClO_4$, most likely due to the salting out effect of ClO_4^-.

The retention behavior in the presence of LiCl, NaCl, and KCl was also compared (Fig. 5) for cation effect study. Because in a cation-exchange process, K^+ is a stronger competing cation than Na^+, which is stronger than Li^+, retention for ephedrine followed the order $K^+ < Na^+ < Li^+$ at same salt concentration. Conversely, salicylic acid was not significantly influenced by change in cation, thus gave three closely positioned retention-salt concentration curves. Surprisingly, tyrosine was not as sensitive as ephedrine to different cations – although exhibiting typical ion-exchange processes, all three retention-salt concentration curves were very close. It was also observed that cation type had no effect on the retention of naphthalene (neutral).

3.2.3. pH effect

Mobile phase pH is another determining factor for retention. The retention behavior was studied at pH 2.3 and pH 6.5 of different buffer concentrations. The pH 2.3 phosphate buffers were prepared by titrating NaH_2PO_4 with H_3PO_4, while titration with Na_2HPO_4 with H_3PO_4 was used for preparing the pH 6.5 buffers. Salt concentrations were all normalized to Na^+. As shown in Fig. 6, ephedrine exhibited virtually overlapping retention-buffer concentration curves at pH 2.3 and pH 6.5. It is because at both pHs, ephedrine (p*K*a = 9.6) was fully positively charged at both pHs and the strong cation-exchange capacity was unaffected by pH change. By comparison, the retention of salicylic acid (p*K*a = 2.97) is more complicated. First, its carboxylic group was fully ionized (more hydrophilic) at pH 6.5 and partially charged (more hydrophobic) at pH 2.3. Secondly, although the weak anion-exchange group (p*K*a ~ 10) was fully charged at both pH levels so that its anion-exchange was considered "constant", free silanols on silica surface also played a role in the process: most silanols are protonated (neutral) at pH 2.3 and most deprotonated (negatively charged) at pH

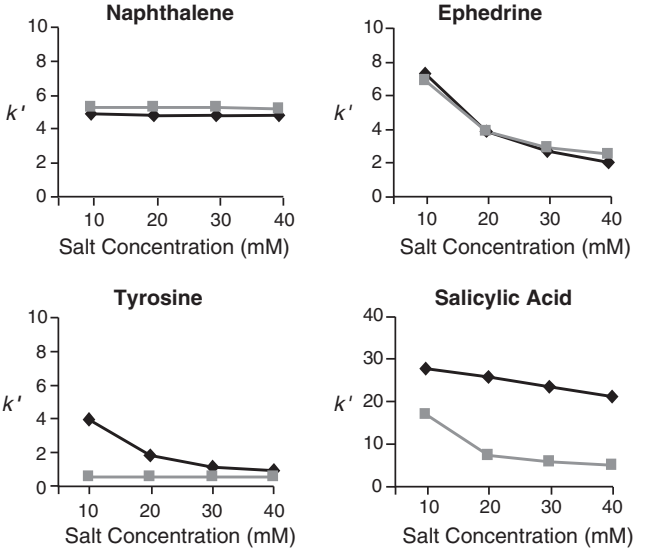

Fig. 6. Effect of pH. Column, trimode phase **A**, 4.6 × 50-mm format; mobile phase, acetonitrile/5–40 mmol L^{-1} (total concentration) sodium phosphate buffer (40:60, v/v); flow rate, 1.0 mL min^{-1}; injection volume, 3 μL; temperature, 30 °C; and detection, UV at 210 nm. Mobile phase pH: pH 2.3 (diamond) and 6.5 (square).

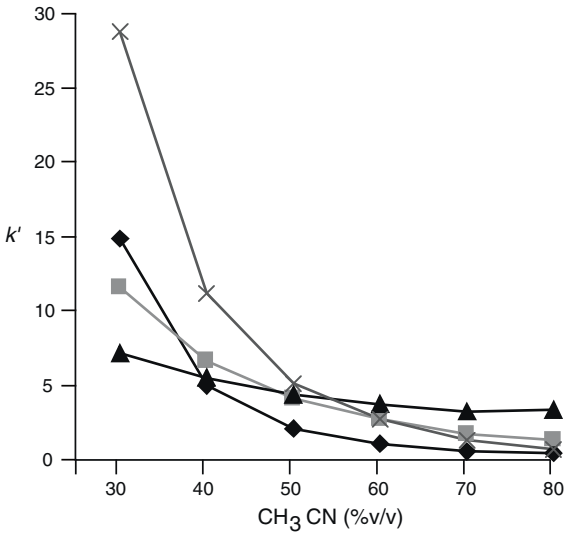

Fig. 7. Effect of organic solvent. Column, trimode phase **A**, 4.6 × 50-mm format; mobile phase, acetonitrile/10 mmol L^{-1} (total concentration) NaCl in 1% H_3PO_4 in various ratios; flow rate, 1.0 mL min^{-1}; injection volume, 3 μL; temperature, 30 °C; and detection, UV at 210 nm. Analytes: naphthalene (diamond), salicylic acid (cross), ephedrine (square), tyrosine (triangle).

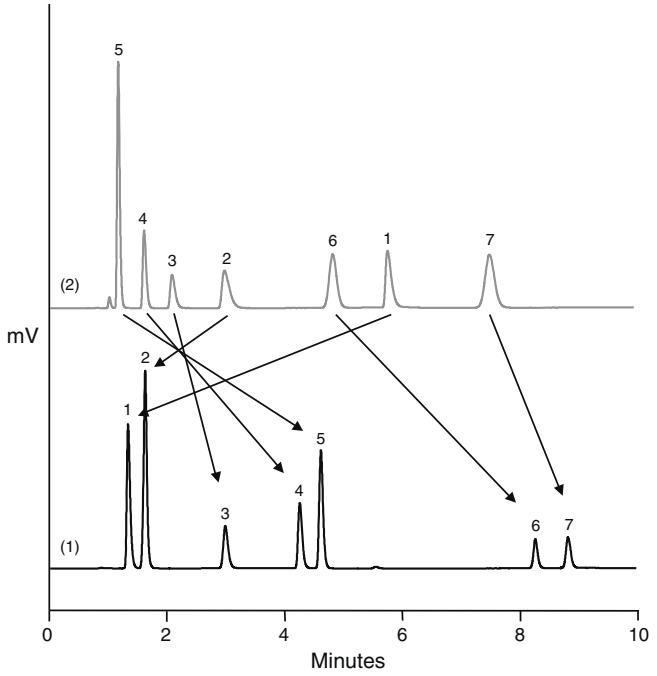

Fig. 8. Separation of alkyl quaternary amines. Column, trimode phase **A**, 3.0 × 50-mm format; mobile phase, A – acetonitrile, B – D.I. water, C – 200 mmol L^{-1} NH_4OAc, pH 5; flow rate, 0.425 mL min^{-1}; injection volume, 2 μL; temperature, 30 °C; and detection, evaporative light scattering detector; sample concentration: 0.2 mg mL^{-1} each. Peaks: (1) $(CH_3CH_2CH_2CH_2CH_2)_4N^+$; (2) $(CH_3CH_2CH_2CH_2)_4N^+$; (3) $(CH_3CH_2CH_2)_4N^+$; (4) $(CH_3CH_2)_4N^+$; (5) $(CH_3)_4N^+$; (6) Cl^-; (7) Br^-. Gradient for (1), ramp from 40%A/57.5%B/2.5%C to 40%A/40%B/20%C in 5 min, then to 40%A/10%B/50%C in 5 min. Gradient for (2), ramp from 10%A/50%B/40%C to 60%A/0%B/40%C in 10 min.

6.5. As the result, the apparent anion-exchange capacity of the stationary phase was higher at pH 2.3 than pH 6.5. Thirdly, at pH 6.5, the phosphate ions were in $HPO_4^{2-}/H_2PO_4^-$ forms and provided stronger elution power compared to that at pH 2.3, in which the phosphate ion was in the form of $H_2PO_4^-$. Therefore, the retention of salicylic acid is determined by ionization of salicylic acid, surface silanols, and phosphate ions at different pH levels. The net outcome is that salicylic acid showed much higher retention at pH 2.3 compared to that at pH 6.5. Tyrosine showed no retention dependency of buffer concentration at pH 6.5, most likely due to the fact that it was neutral under this condition. At pH 2.3, on the other hand, its retention decreased with the increase of buffer concentration, indicating a typical cation-exchange behavior. Naphthalene (neutral) exhibited slightly higher retention at pH 6.5 as opposed to that at pH 2.3, probably because the stationary phase was more hydrophobic at higher pH as the result of the less charged weak anion-exchange sites.

3.2.4. Organic solvent effect

Fig. 7 shows that at a given salt concentration, all analytes were less retentive with an organic solvent increase. In addition, elution order (selectivity) changed with organic solvent content. Naphthalene was affected most by solvent content as it was retained solely by hydrophobic retention. Salicylic acid had slight negative charge under the test condition (pH 2.3), thus it was retained mostly by hydrophobic interaction, superimposed by some anion-exchange interactions. Its retention was more affected by solvent content compared to the fully charged analyte, such as ephedrine, in which case the ion-exchange process contributed more to retention. A possible explanation is that the ionization of the carboxylic group in salicylic acid is more sensitive towards mobile phase organic solvent.

Both ephedrine and tyrosine are fully ionized at pH 2.3, thus are retained by both hydrophobic and cation-exchange interactions. Therefore, their retentions were less sensitive to solvent change, and decreased at a slower rate with the increase of solvent content. Below 50% acetonitrile, tyrosine exhibited lower retention than ephedrine. Above 50% acetonitrile, tyrosine became more retentive than ephedrine. Above 70% acetonitrile, the k' of tyrosine started to increase with solvent content – an indication of a hydrophilic interaction process [15].

3.3. Applications

3.3.1. Separation of alkyl quaternary amines in RP and IEX modes

Fig. 8(1) illustrates four alkyl quaternary amines (quats) and their inorganic counterions simultaneously separated by cation-exchange superimposed by reversed-phase retention on a 3.0 × 50-mm column packed with phase **A**. The chromatographic method consisted of a salt gradient while keeping acetonitrile constant at 40% (v/v). The elution order followed the charge density (defined as the number of charge divided by the size of the molecule) of the analyte – larger quats (lower charge density) eluted before smaller ones. In the same analysis, inorganic counterions (chloride and bromide) were also separated by anion-exchange, and eluted at higher buffer concentration. Fig. 8(2) shows the separation of the same sample using a different chromatographic condition in which an organic solvent gradient was used at a constant salt concentration (80 mmol L^{-1}). Quats were separated by reversed-phase mechanism superimposed by a cation-exchange process. The elution order followed the hydrophobicity of analyte – more hydrophobic ones were more retentive than less hydrophobic ones. In addition, counterions (chloride and bromide) were separated from quats during the same analysis by anion-exchange interactions.

3.3.2. Separation of pharmaceutical counterions

Salt formation is a critical step in drug development because it provides improved biopharmaceutical and physicochemical properties, ease of purification and handling [16,17]. According to the Orange Book database published by the U.S. Drug and Food Administration (FDA), among the 1356 API (Active Pharmaceutical Ingredient) approved by FDA up to the end of 2006, 51.4% are in salt forms, with 38.6% formed from basic molecules and

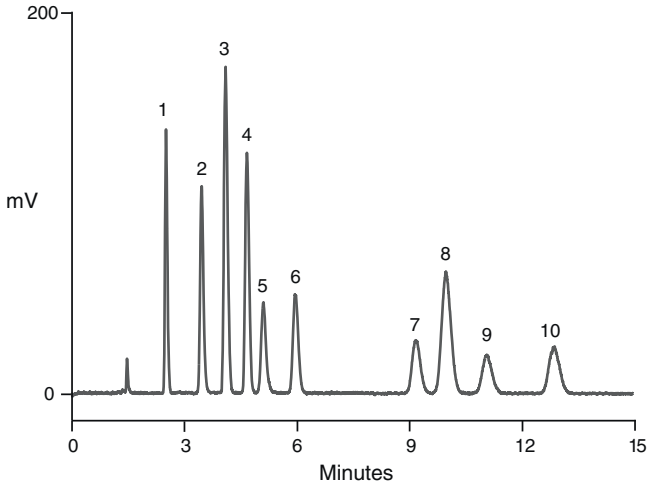

Fig. 9. Separation of pharmaceutical counterions. Column, Acclaim Trinity P1, 3.0 × 100-mm format; mobile phase, acetonitrile/ammonium acetate buffer, pH 5 (20 mmol L^{-1} total concentration) (60:40, v/v); flow rate, 0.5 mL min^{-1}; injection volume, 2 μL; temperature, 30 °C; and detection, Corona *ultra* (gain = 100 pA; filter = med; nebulizer temperature = 30 °C). Sample: 0.05 to 0.1 mg mL^{-1}). Peaks: (1) Choline, or *N*,*N*,*N*-trimethylethanolammonium; (2) tromethamine, or tris(hydroxymethyl)aminomethane; (3) sodium; (4) potassium; (5) meglumine, or N-methyl glucamine; (6) mesylate, or methanesulfonate; (7) nitrate; (8) chloride; (9) bromide; (10) iodide.

12.8% formed from acidic molecules [18,19]. The most commonly used counterions are sodium and chloride ions. No reversed-phase columns can retain or separate either sodium or chloride. Although an ion-exchange column can retain sodium or chloride, coupling of one anion-exchanger and one cation-exchanger is required to separate both ions in a single analysis, which increases the method complexity, reliability and cost. The reversed-phase/anion-exchange/cation-exchange trimode technology offers a solution to this challenge. A broad selection of inorganic and organic ions can be used as pharmaceutical counterions [17]. It is highly desirable to separate both pharmaceutically important anions and cations within the same analysis and in a reasonable amount of time. The optimal selectivity can be achieved by careful balancing between the anion-exchange capacity and cation-exchange capacity. It was found that although both Na^+ and Cl^- ions were separated on phase **A** (based on 300 m^2/g, 120 Å, 5-μm silica gel), Cl^- was approximately ten times more retentive than Na^+, suggesting an excessive anion-exchange capacity. To reduce anion-exchange capacity for the desirable selectivity, we prepared phase **B** using a silica gel of lower surface area (100 m^2/g), larger pore size (300 Å), and smaller particle size 3-μm). The resulting phase exhibited improved selectivity (k'_{Cl}/k'_{Na}~3) and higher peak efficiency compared to phase **A**. As shown in Fig. 9, phase **B** provided good selectivity for separating pharmaceutical counterions using acetonitrile/ammonium acetate mobile phase: baseline separation of 10 commonly used pharmaceutical counterions (five cations and five anions) was obtained within a single run. The phase **B** also exhibited suitable chromatographic properties for simultaneous separation of drug molecules and their counterions (not shown). For this reason, phase **B** was commercialized [20,21] and has been successfully used for pharmaceutical counterion analysis [22] and for melamine analysis in dairy products and pet foods [23].

4. Concluding remarks

New reversed-phase/anion-exchange/cation-exchange trimodal stationary phases were developed. They are based on high-purity, spherical, porous silica particles which inner-pore area was functionalized with an organic layer that provides both reversed-phase and anion-exchange properties while the outer surface is coated with cation-exchange functionality through electrostatic attractions. One chromatographic feature is the capability of providing simultaneous reversed-phase, anion-exchange, and cation-exchange retention so that neutral, acidic, and basic analytes can be separated within a single analysis. Another feature is the adjustable selectivity that can be controlled by mobile phase composition, such as buffer concentration, salt type, pH and organic solvent concentration. It has been demonstrated that these trimodal phases can be used for separating alkyl quaternary amines and pharmaceutical counterions.

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