Anion-exchange chromatography on selected silica-based reversed-phase high-performance liquid chromatographic columns

STEPHEN L. SECREAST

The Upjohn Company, Control Division, 4821-259-12, 7000 Portage Road, Kalamazoo, MI 49001 (U.S.A.)

ABSTRACT

An unexpected retention of nitrate was observed on a Zorbax Rx C_8 high-performance liquid chromatographic column suggesting an anion-exchange retention mechanism. The ability of the column to be an anion exchanger was investigated using aqueous phosphoric and acetic acid mobile phases. For comparison Nucleosil C_8 and Zorbax C_8 columns were also examined. Significant retention and separation of anions was achieved on the Zorbax Rx C_8, to some extent on the Nucleosil C_8, but not on the Zorbax C_8. Taking advantage of the unusual anion-exchange property, the Zorbax Rx C_8 column was used to simultaneously chromatograph the amine plus counter-anion components of some research pharmaceutical salts by a mixed-mode reversed-phase anion-exchange mechanism.

INTRODUCTION

Anions are generally believed to have little interaction with silica-based reversed-phase packing materials, as evidenced by their use as void volume markers [1]. What little interaction that does occur is believed to be pH dependent ion exclusion due to dissociated silanol sites on the silica supports [1]. The pK_a of silica ($\equiv SiO^- \leftrightarrow \equiv SiOH$) has been determined [2] to be about 7. The isoelectric point, the point where all the silanols are uncharged ($\equiv SiOH$), has been determined [3] to be at about pH 2. With typical mobile phases with a pH $\geqslant$ 2, the packing materials can have a net negative charge, causing anion exclusion.

Anion retention, in the absence of amine modifiers and metal contaminants, with mobile phases of pH $\geqslant$ 2, is not expected [4]. Recently, however, during development of a separation on the relatively new Zorbax Rx C_8 column (250 × 4.6 mm I.D.), using an aqueous acetonitrile–phosphoric acid mobile phase of apparent pH 3.1, inorganic nitrate was observed to elute at about 10 min. These results suggested retention by an ion-exchange mechanism. In this work, as a follow-up on this unexpected observation, anion-exchange chromatography on a Zorbax Rx C_8 column was studied. For comparison Nucleosil C_8 and Zorbax C_8 columns were also investigated.

EXPERIMENTAL

Chromatographic conditions

All chromatography was performed on an HP1090M integrated high-perform-ance liquid chromatograph consisting of an HP79835A ternary solvent delivery system, an HP79846A variable volume auto-injector, an HP79847B auto-sampler, an HP1040A diode array detector, and an HP79994A Chemstation (Hewlett-Packard, Palo Alto, CA, U.S.A.). The columns used were a Zorbax C_8, 250 × 4.6 mm I.D. (DuPont, Wilmington, DE, U.S.A.) a Nucleosil C_8, 5 μm, 100 Å, 250 × 4.6 mm I.D. [Machery-Nagel, Düren, Germany (packed by Alltech Associates, Deerfield, IL, U.S.A.)], and a Zorbax Rx C_8, 250 × 4.6 mm I.D. (DuPont, Wilmington, DE, U.S.A.). Detection was by UV at 205 or 214 nm. Injection volumes were 10 μl. The flow-rate was 1.0 ml/min in all cases. The mobile phases used were aqueous solutions of phosphoric acid, aqueous solutions of acetic acid plus sodium hydroxide (to adjust pH), and the same solutions with acetonitrile.

Reagents

High purity water, > 16 MΩ-cm, (Millipore, Bedford, MA, U.S.A.), HPLC-grade acetonitrile (Burdick and Jackson, Muskegon, MI, U.S.A.), and analytical reagent grade glacial acetic acid and 85% phosphoric acid (Mallinckrodt, Paris, KE, U.S.A.) were used.

Anions

Solutions of analytical reagent grade sodium nitrate, 0.006 mg/ml (J. T. Baker, Phillipsburg, NJ, U.S.A.); potassium bromide, 0.012 mg/ml; potassium thiocyanate, 0.020 mg/ml; potassium iodide, 0.015 mg/ml; and potassium iodate, 0.030 mg/ml (Mallinckrodt, Paris, KE, U.S.A.) were used as a source of anions.

Pharmaceutical compounds

U-80,042 hydrobromide salt, Nicorandil (free base), Nicorandil fumaric acid salt, and U-75,412 maleic acid salt were obtained from The Upjohn Company and were chromatographed as 0.2, 1.0, 0.1 and 0.1 mg/ml solutions, respectively.

RESULTS AND DISCUSSION

Nitrate chromatography

Initially, nitrate was chromatographed on a new Zorbax C_8 column (SN L15972), a new Nucleosil C_8 column (Alltech SN 01260QA), and a new Zorbax Rx C_8 column (SN AU5158), using aqueous phosphoric acid and acetic acid mobile phases and detection at 205 nm. The results for the phosphoric acid mobile phases are shown in Table I. With the pH 3.2, 0.7 mM $H_2PO_4^-$ mobile phase, nitrate had a 1.76-min retention time on the Zorbax C_8 column, indicating elution at or very near the column void, as expected. However, nitrate eluted at 7.50 min on the Nucleosil C_8 column, and 10.59 min on the Zorbax Rx C_8 column. As the mobile phase $H_2PO_4^-$ concentration was increased, nitrate retention on the Zorbax C_8 column was unchanged, but decreased on the Nucleosil C_8 and Zorbax Rx C_8 columns. With the last mobile phase, sodium chloride was added as an additional source of counter-ions, further decreasing

nitrate retention on the Nucleosil C_8 and Zorbax Rx C_8 columns. These results suggested an anion-exchange retention mechanism for nitrate on the Nucleosil C_8 and Zorbax Rx C_8 columns.

The results for the aqueous acetic acid mobile phases are shown in Table II. The acetic acid concentrations of these mobile phases were selected so that when the mobile phase pH was adjusted to the values listed, the mobile phase acetate concentration would be 0.1 mM (assuming the pK_a = 4.75). With the pH 4.0, 0.1 mM acetate mobile phase on the Zorbax C_8 column, nitrate had a 1.67-min retention time, which remained constant with increasing pH and was similar to the Table I results. With this mobile phase, however, nitrate eluted at 2.70 min on the Nucleosil C_8 column and 17.15 min on the Zorbax Rx C_8 column. Increasing mobile phase pH, while maintaining constant acetate concentration, decreased nitrate retention on the Nucleosil C_8 column until pH 5.0–5.5 where the retention time leveled off. On the Zorbax Rx C_8 column, nitrate retention steadily decreased and at pH 6.0 was still greater than that seen on the other two columns. These results again suggested an anion-exchange retention mechanism for nitrate on the Nucleosil C_8 and Zorbax Rx C_8 columns. The number of cationic sites on the columns appears to be pH dependent, with the Zorbax Rx C_8 material being the most basic, followed by the Nucleosil C_8. Under these conditions, the Zorbax C_8 does not develop a significant cationic surface.

Multiple anion chromatography

To assure that the retention observed for nitrate was not an occurrence unique to that one anion, a mixed sample of iodate, bromide, nitrate, iodide, and thiocyanate was chromatographed on the three columns with detection at 205 nm. Fig. 1 shows the chromatograms obtained using an aqueous 1.5 mM phosphoric acid, pH 2.9 mobile phase. Fig. 2 shows the chromatograms obtained using an aqueous 0.2 mM acetic acid, pH 5.0 mobile phase. The results again showed both the Nucleosil C_8 and the Zorbax Rx C_8 columns were good anion exchangers at pH 2.9 with aqueous 1 mM $H_2PO_4^-$ as an eluent. Only the Zorbax Rx C_8 column was still a good anion exchanger at pH 5.0 with aqueous 0.1 mM acetate as an eluent.

TABLE I

NITRATE RETENTION USING AQUEOUS PHOSPHORIC ACID MOBILE PHASES

$H_3PO_4^a$ (mM)	pH[b]	$H_2PO_4^{-c}$ (mM)	Nitrate retention time (min)		
			Zorbax C_8	Nucleosil C_8	Zorbax Rx C_8
0.7	3.2	0.7	1.76	7.50	10.59
1.5	2.9	1.3	1.73	5.85	5.92
7.4	2.4	5.3	1.74	4.36	3.85
14.7	2.2	8.5	1.74	4.08	3.51
14.7[d]	2.2	8.5[e]	not run	3.28	2.90

[a] Concentration of phosphoric acid in water to make mobile phase.
[b] Measured mobile phase pH.
[c] Theoretical mobile phase anion concentration (assuming $H_3PO_4 \leftrightarrow H_2PO_4^-$, p$K_a$ = 2.1).
[d] +20 mM NaCl.
[e] +20 mM Cl$^-$.

TABLE II

NITRATE RETENTION USING AQUEOUS ACETIC ACID MOBILE PHASES

Acetic acid[a] (mM)	pH[b]	Acetate[c] (mM)	Nitrate retention time (min)		
			Zorbax C_8	Nucleosil C_8	Zorbax Rx C_8
0.7	4.0	0.1	1.67	2.70	17.15
0.3	4.5	0.1	1.66	2.12	11.37
0.2	5.0	0.1	1.65	1.86	7.77
0.1	5.5	0.1	1.66	1.70	4.22
0.1	6.0	0.1	1.65	1.76	2.99

[a] Concentration of acetic acid in water to make mobile phase.
[b] pH mobile phase adjusted to using aqueous 1 M sodium hydroxide.
[c] Theoretical mobile phase anion concentration (assuming $pK_a = 4.75$).

Effect of acetonitrile on anion-exchange chromatography

The ability to perform anion-exchange chromatography on silica-based reversed-phase columns is of limited usefulness, and certainly not the most efficient method available for anions. However, the ability to perform mixed-mode reversed-phase anion-exchange chromatography is potentially more useful. To assess the ability of the columns to be anion exchangers in the presence of organic solvent (an obvious requirement for performing the mixed-mode chromatography), acetonitrile was added to the aqueous acidic mobile phases and the anion mix described above was again chromatographed.
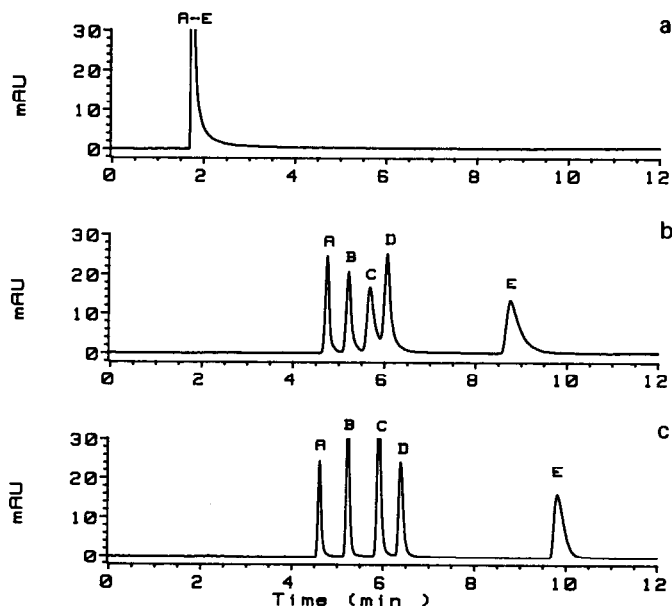

Fig. 1. Anion-exchange chromatography with an aqueous 1.5 mM phosphoric acid, pH 2.9 mobile phase. (a) Zorbax C_8; (b) Nucleosil C_8 and (c) Zorbax Rx C_8. Peaks: A = iodate, B = bromide, C = nitrate, D = iodide, E = thiocyanate.
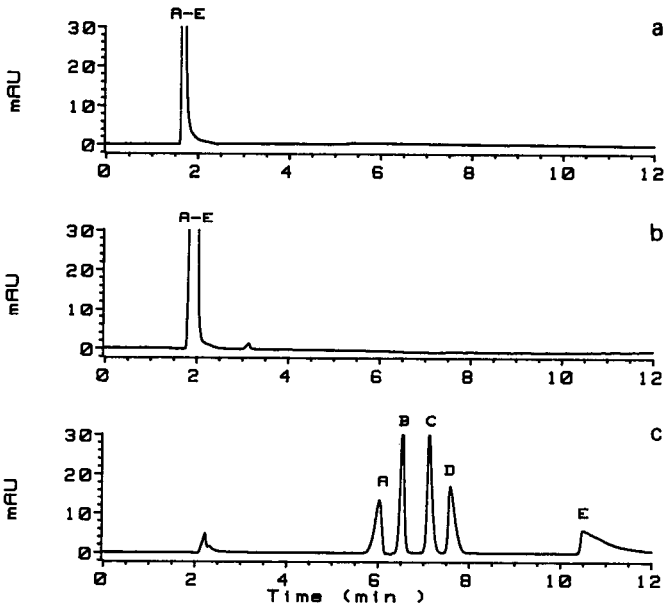
Fig. 2. Anion-exchange chromatography with an aqueous 0.2 mM acetic acid, pH 5.0 mobile phase. Chromatogram key and peak key as for Fig. 1.
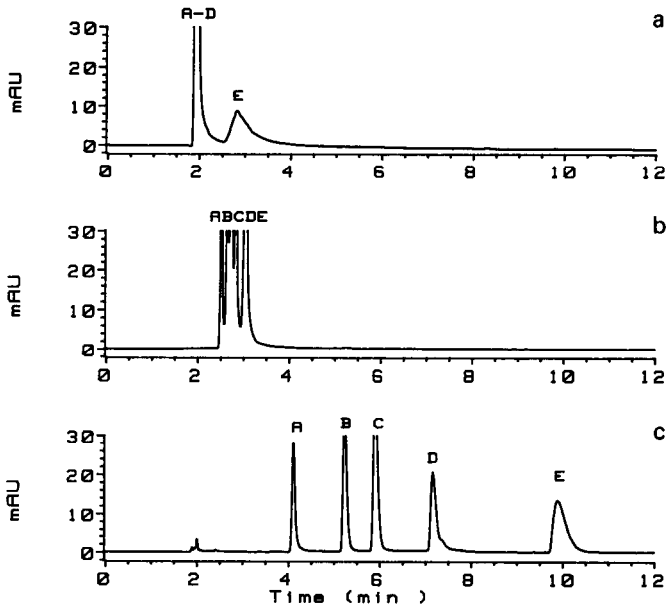

Fig. 3. Anion-exchange chromatography with a 25% acetonitrile–1.5 mM phosphoric acid, apparent pH 2.9 mobile phase. Chromatogram key and peak key as for Fig. 1.

Fig. 3 shows the chromatograms obtained using a 25% acetonitrile–1.5 mM phosphoric acid, apparent pH 2.9 mobile phase. The anion-exchange ability of the Nucleosil C_8 column was significantly hindered by the addition of acetonitrile to the mobile phase whereas the Zorbax Rx C_8 column changed very little. These results again suggest that column basicity is in the order of Zorbax Rx C_8 > Nucleosil C_8 > Zorbax C_8. It is interesting to note that the retention of the anions, most notably thiocyanate, increased slightly on the Zorbax C_8 column. This indicates that ion exclusion occurs with the aqueous acidic mobile phases, and is hindered by the addition of acetonitrile, allowing some hydrophobic interaction then to occur.

Fig. 4 shows the chromatograms obtained using a 25% acetonitrile–0.2 mM acetic acid, apparent pH 5.0 mobile phase. At this pH, acetonitrile completely hindered the anion-exchange ability of the Nucleosil C_8 column, and also significantly hindered the Zorbax Rx C_8 column. These results indicate that mixed-mode reversed-phase anion-exchange chromatography can best be performed on the Zorbax Rx C_8 column at or near pH 3, but would be more difficult at higher pH.

Mixed-mode reversed-phase anion-exchange application

A number of research pharmaceutical compounds (Fig. 5) were chromatographed on the Zorbax Rx C_8 column using mixed-mode conditions. The chromatogram of the hydrobromide salt of the organic amine U-80,042 is shown in Fig. 6. With detection at 205 nm and a 25% acetonitrile–0.7 mM phosphoric acid, apparent pH 3.1 mobile phase, the compound plus the bromide counter-ion were chromatographed simultaneously with bromide eluting after the amine. The chromatogram of another compound, Nicorandil (free base), is shown in Fig. 7. This compound is nicotinamide
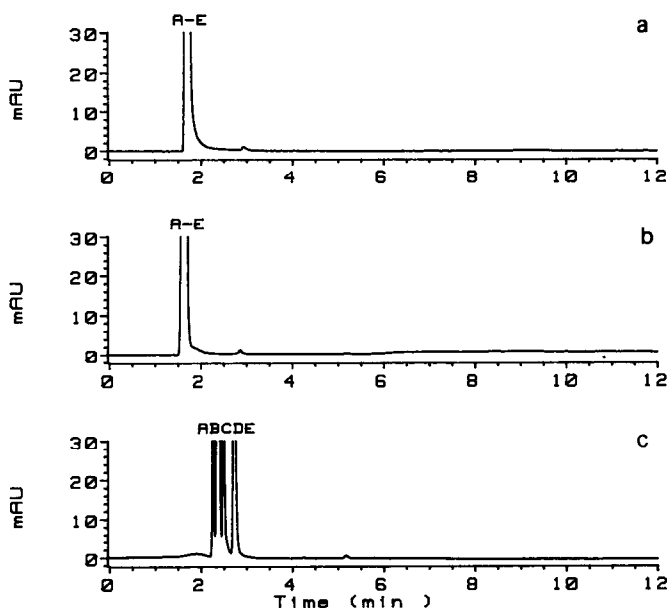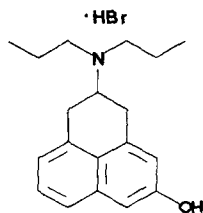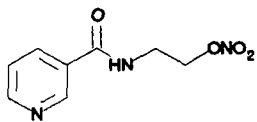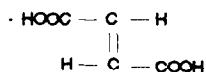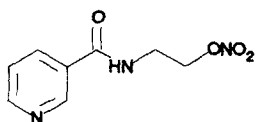

Fig. 4. Anion-exchange chromatography with a 25% acetonitrile–0.2 mM acetic acid, apparent pH 5.0 mobile phase. Chromatogram key and peak key as for Fig. 1.
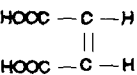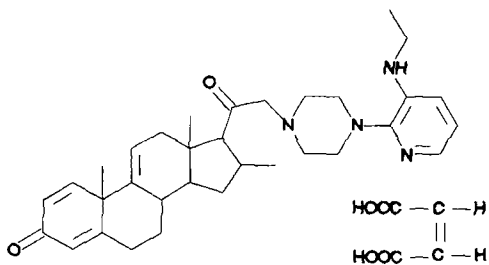
Fig. 5. Structures of research pharmaceutical compounds chromatographed by mixed-mode reversed-phase anion-exchange HPLC on a Zorbax Rx C$_8$ column. Structure key: A = U-80,042 hydrobromide salt, B = Nicorandil (free base), C = Nicorandil fumaric acid salt, D = U-75,412 maleic acid salt.
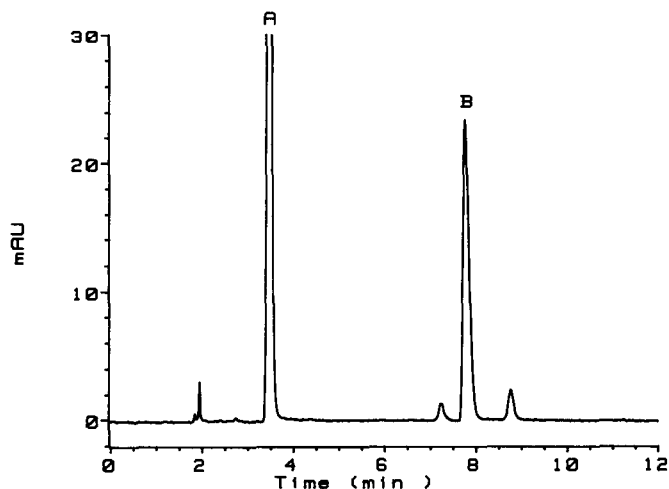
Fig. 6. Mixed-mode reversed-phase anion-exchange chromatography of U-80,042 hydrobromide salt. Column: Zorbax Rx C$_8$. Detection: 205 nm. Mobile phase: 25% acetonitrile–0.7 mM phosphoric acid, apparent pH 3.1. Peaks: A = U-80 042, B = bromide.

based with a nitrate ester functionality. It degrades to form amines plus inorganic nitrate [5]. Nicorandil, the amine degradation products, and the nitrate degradation product were chromatographed simultaneously with a 30% acetonitrile–0.7 mM phosphoric acid, apparent pH 3.1 mobile phase, 205 nm detection, with nitrate eluting last.

Compounds with organic acid counter-anions were also successfully chromatographed. Best results were obtained with fairly polar amines that did not require high
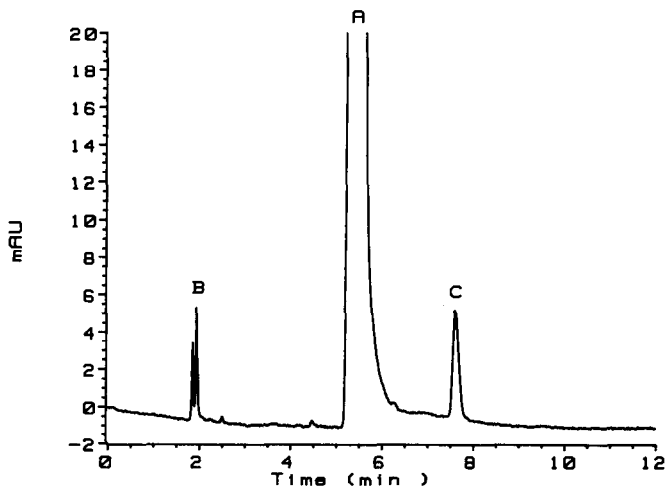

Fig. 7. Mixed-mode reversed-phase anion-exchange chromatography of Nicorandil (free base). Column: Zorbax Rx C$_8$. Detection: 205 nm. Mobile phase: 30% acetonitrile–0.7 mM phosphoric acid, apparent pH 3.1. Peaks: A = Nicorandil, B = amine degradation products, C = nitrate.
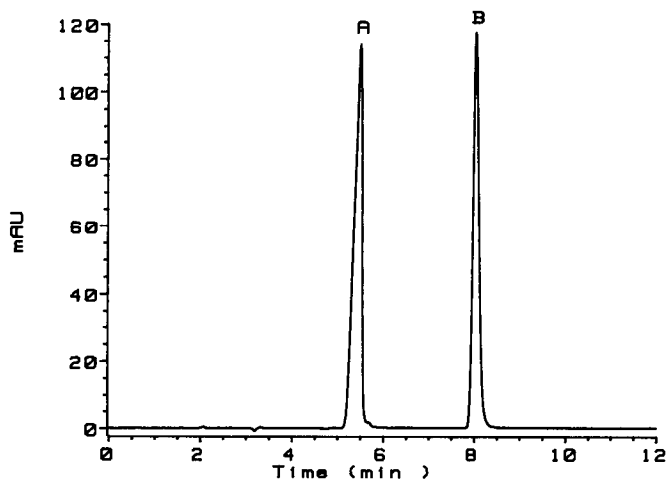
Fig. 8. Mixed-mode reversed-phase anion-exchange chromatography of Nicorandil fumaric acid salt. Column: Zorbax Rx C_8. Detection: 214 nm. Mobile phase: 25% acetonitrile–0.2 mM acetic acid, apparent pH adjusted to 4.3. Peaks: A = fumarate, B = Nicorandil.

concentrations of acetonitrile for elution, and organic acid counter-anions of $pK_a < 4.5$ (in 25–40% acetonitrile). If higher acetonitrile concentrations were required or less acidic counter-anions were used, requiring higher pH mobile phases, inhibition of cationic column surface formation became significant enough to prevent anion-exchange chromatography. Fig. 8 shows the chromatogram of the fumaric acid salt of Nicorandil with a 25% acetonitrile–0.2 mM acetic acid, apparent pH 4.3 mobile phase and detection at 214 nm. The first pK_a of fumaric acid [6] is 3.0. At pH 4.3 with 25%
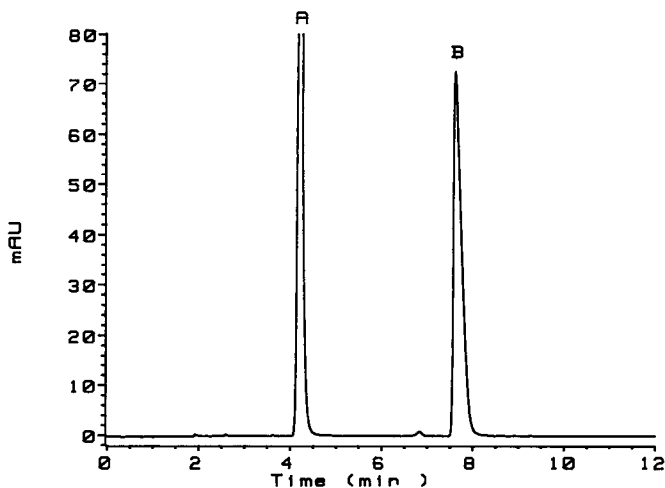

Fig. 9. Mixed-mode reversed-phase anion-exchange chromatography of U-75,412 maleic acid salt. Column: Zorbax Rx C_8. Detection: 214 nm. Mobile phase: 40% acetonitrile–0.7 mM phosphoric acid, apparent pH 3.1. Peaks: A = U-75,412, B = maleate.

acetonitrile the column is less cationic than with the pH 3.1 mobile phases and fumarate eluted at about 5 minutes. Fig. 9 shows the chromatogram of the maleic acid salt of the amine U-75,412 with a 40% acetonitrile–0.7 mM phosphoric acid, apparent pH 3.1 mobile phase and detection at 214 nm. The first pK_a of maleic acid [6] is 1.8. Even with 40% acetonitrile, the Zorbax Rx column at pH 3.1 is cationic enough for maleate to be retained almost 8 minutes.

　　Using U-75,412 maleic acid salt as a model compound, the effects of mobile phase acetonitrile concentration, phosphoric acid concentration, and addition of chloride (as sodium chloride) on the mixed-mode chromatography were investigated. Results are shown in Table III. Decreased mobile phase acetonitrile concentration with constant H_3PO_4 concentration resulted in significantly increased amine retention while having little effect on counter-anion retention. Conversely, increased mobile phase H_3PO_4 concentration with constant acetonitrile concentration resulted in little effect on amine retention, but significantly decreased retention of the counter-anion. The addition of chloride to the acetonitrile–H_3PO_4 mobile phase significantly increased the retention of the amine and also decreased the retention of the anion.

　　Taken together, these results indicate that retention of the amine is due predominantly to reversed-phase interactions. However, as the addition of chloride caused increased amine retention, ion-exclusion interactions with the cationic column surface may also play a role. The results also indicate retention of the counter-anion is due predominantly to anion-exchange interactions.

Zorbax Rx C$_8$ column-to-column anion-exchange reproducibility

　　The column-to-column reproducibility of anion-exchange chromatography on Zorbax Rx C$_8$ columns was assessed using nitrate as a probe and 25% acetonitrile–0.7 mM phosphoric acid as a mobile phase. Nitrate retention time on a number of

TABLE III

EFFECTS OF ORGANIC SOLVENT STRENGTH AND IONIC STRENGTH ON ZORBAX Rx C$_8$ MIXED-MODE CHROMATOGRAPHY

t_R = Retention time (min).

Constant[a] H$_3$PO$_4$			Constant[b] acetonitrile			Constant[c] acetonitrile + H$_3$PO$_4$		
% acetonitrile	Amine t_R	Anion t_R	mM H$_3$PO$_4$	Amine t_R	Anion t_R	mM Cl$^-$	Amine t_R	Anion t_R
50.0	2.59	7.95	0.7 (pH 3.1)	3.89	7.85	0	3.94	7.55
40.0	4.22	7.62	7.0 (pH 2.6)	3.62	4.99	0.5	5.16	5.33
34.0	9.01	7.70	70.0 (pH 2.1)	3.30	3.30	1.5	6.22	3.96

[a] Mobile phase = aqueous 0.7 mM H$_3$PO$_4$, apparent pH 3.1 with acetonitrile as indicated.
[b] Mobile phase = aqueous 40% acetonitrile with H$_3$PO$_4$ as indicated.
[c] Mobile phase = aqueous 40% acetonitrile–0.7 mM H$_3$PO$_4$, apparent pH 3.1 with Cl$^-$ (as NaCl) as indicated.

250 × 4.6 mm I.D. columns is shown in Table IV. A significant change in nitrate retention was observed between columns AU 1922 and AU 2648 and again between columns AU 2932 and AU 5138 suggesting column acidity increased somewhere within the two ranges. The latter observed change corresponds to a process modification made by the column manufacturer, starting with column AU 5000, which apparently did slightly increase column acidity [7]. These results indicate mobile phase pH and ionic strength adjustments may be necessary to achieve reproducible column-to-column anion retention. The results also indicate nitrate is a sensitive probe for column-to-column acidity variations.

Possible cause of the anion-exchange chromatography

Without knowing exactly how the Zorbax C_8, Nucleosil C_8, or Zorbax Rx C_8 packing materials were made, one could attribute the ability (or lack of ability) of the materials to be anion exchangers to several possible causes. The presence (or absence) of metal impurities, amine polymers, or amine spacers could all potentially be responsible for the observed anion-exchange chromatography. Another interesting possibility, however, is that the silica supports themselves could be the source of the cationic sites.

The three columns used in this study have previously been ranked by acidity [8] as: Zorbax C_8 > Nucleosil C_8 > Zorbax Rx C_8. The ability of the materials to be anion-exchangers, like the degree of acidity, could possibly be due to silanol chemistry. Silanol groups are classified as two major types: isolated silanols and associated silanols [9]. Previous work [10,11] has determined that isolated silanols are highly acidic and may be responsible for problematic chromatography of amines. Silica with more associated silanols was found to be less acidic and, therefore, a more desirable support for reversed-phase HPLC. The work concluded that Nucleosil silica is less acidic than Zorbax silica because it has more associated silanols (has fewer isolated sites). Ultimately this work [10,11] led to the development of Zorbax Rx silica having the most associated silanols of the three. If associated silanols are less acidic than isolated silanols, they may also conceivably be more basic, that is, they may have a higher $\equiv SiOH \leftrightarrow \equiv Si\text{-}OH_2^+$ pK_a.

TABLE IV

NITRATE RETENTION REPRODUCIBILITY ON ZORBAX Rx C_8 COLUMNS

Mobile phase: 25% acetonitrile–0.7 mM phosphoric acid.

Column serial number	Nitrate t_R[a] (min)
AU 1707	15.8
AU 1922	16.7
AU 2648	11.3
AU 2932	11.6
AU 5158	5.9
AU 5262	5.6
AU 5393	3.6
AU 5394	3.6

[a] Value is the calculated mean of three injections.

Previous work [12] has shown it necessary to achieve pH levels of <2 to make silica cationic. That appears to be the case for Zorbax silica, but not for more silanol-associated silicas such as that used in Zorbax Rx. What could exist on the silica surface at pH 2–7 is a mix of anionic-to-neutral isolated silanol sites plus neutral-to-cationic associated silanol sites. At a given pH the net surface charge of a given silica-based reversed-phase packing material would be determined, then, by the ratio of the amount of associated silanols to the amount of isolated silanols. With the pH 2.9–5.0 aqueous acidic mobile phases used here, the Zorbax silica having mostly isolated silanols would have a net negative charge. Nucleosil silica, having more associated silanols, would have a slight net positive charge, and the Zorbax Rx silica, having yet more associated silanols, would have a greater net positive charge.

The addition of acetonitrile to the mobile phases shifts the $\equiv SiOH \leftrightarrow \equiv SiOH_2^+$ pK_a to a lower value making the silicas more neutral at the same pH levels. Zorbax Rx silica, having the greatest number of associated silanols, could best remain cationic under these conditions, allowing mixed-mode reversed-phase anion-exchange chromatography.

This chemistry could explain why amines are adsorbed less on the Zorbax Rx C_8 and the Nucleosil C_8 columns, compared to the Zorbax C_8 column, as a result of electrostatic repulsion from a cationic silica surface. This would also explain why the Nucleosil C_8 and Zorbax Rx C_8 can be anion-exchangers. Further analysis [10,11] of these and other silica-based materials by IR, NMR, titration, or other means would be needed to confirm or disprove this hypothesis.

CONCLUSIONS

Anion-exchange chromatography using aqueous phosphoric and acetic acid mobile phases can be performed on the silica-based Zorbax Rx C_8, to some extent on the Nucleosil C_8, but not to any significant extent on the Zorbax C_8 columns. The Zorbax Rx C_8 column is sufficiently basic to perform mixed-mode reversed-phase anion-exchange HPLC of amines, plus their counter-anions, as demonstrated with several pharmaceutical compounds. Amine and counter-anion retention are greatly effected by mobile phase organic solvent strength, ionic strength, and choice of ionic eluant.

Nitrate retention can be used to examine Zorbax Rx C_8 column-to-column surface acidity reproducibility. Significant changes in column acidity were observed at certain points in the manufacturing history of the column, indicating adjustments in mobile phase pH and ionic strength may be necessary to maintain reproducible column-to-column anion-exchange chromatography.

The hypothesis that associated silanol sites on the silica supports of the column packing materials are sufficiently basic to be made cationic at pH >2 is a possible explanation for the observed anion-exchange chromatography.

REFERENCES

1 P. C. Sadek, P. W. Carr and L. D. Bowers, *Liq. Chromatogr.*, 3 (1985) 590–592.
2 K. K. Unger, *Porous Silica*, Elsevier, Amsterdam, 1979, p. 133.
3 K. K. Unger, *Porous Silica*, Elsevier, Amsterdam, 1979, p. 130.
4 Q. Xianren and W. Baeyens, *J. Chromatogr.*, 456 (1988) 267–285.
5 H. Nagai, M. Kikuchi, H. Nagano and M. Shiba, *Chem. Pharm. Bull.*, 32(3) (1984) 1063–1070.
6 R. C. Weast (Editor), *CRC Handbook of Chemistry and Physics*, CRC Press, Boca Raton, FL, 1980, p. D-165.
7 Personal communication, MAC-MOD Analytical sales representative, March, 1990.
8 L. R. Snyder, J. L. Glajch and J. J. Kirkland, *Practical HPLC Method Development*, John Wiley & Sons, New York, 1988, p. 62.
9 K. K. Unger, *Porous Silica*, Elsevier, Amsterdam, 1979, p. 7.
10 J. Köhler, D. B. Chase, R. D. Farlee, A. J. Vega and J. J. Kirkland, *J. Chromatogr.*, 352 (1986) 275–305.
11 J. Köhler and J. J. Kirkland, *J. Chromatogr.*, 385 (1987) 125–150.
12 K. K. Unger, *Porous Silica*, Elsevier, Amsterdam, 1979, p. 138.