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LIQUID CHROMATOGRAPHY OF POLAR AROMATIC COMPOUNDS ON CATION-EXCHANGE RESINS AND POROUS POLYMER GELS

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SUMMARY

The retention and separation of some 12 polar aromatic compounds, some of them weak acids and ampholytes, have been studied on cation-exchange resins and a non-ionic macroporous styrene–divinylbenzene polymer. The effects of pH, counter-ions and ion-pairing reagents have been measured. Ammonium ions extend the absorption of weak acids (pK_a above 8) to higher pH values on an ion-exchange resin. The porous polymer has more selectivity than the ion-exchange resins, but plate numbers are less. Gradient elution may be used with the porous polymer, but radical solvent changes affect the quality of the packing.

INTRODUCTION

Cation-exchange resins serve as stationary phases for the chromatography of non-ionic organic compounds[1–3] and of weak acids, both aromatic and aliphatic[4–6]. The acids are absorbed by the resin polymer matrix in their non-ionized forms and excluded in their ionized or ionic forms. Retention depends on the ionization constants and the pH of the solution, and in mixtures of acids of differing ionization constants the elution sequence can be changed by changing the pH. "Ion-exclusion chromatography", the separation of acids on a cation-exchange resin at selected pH values, has been used to advantage in the analysis of nucleic acid derivatives[7,8]. Because many of the compounds to be separated have a limited solubility in water, it is customary to use mixtures of water with another solvent, such as alcohol or methyl cellosolve[5]. The composition of the solvent mixture strongly affects the retention and may affect the elution sequence.

Fast mass transfer requires resins of low cross-linking. Polystyrene-based resins with 2%[4] and 4%[2,3,6] divinylbenzene have been used for this type of chromatography. Resins with small particle sizes and low cross-linking give excellent theoretical plate heights, routinely 0.1 mm and less, with symmetrical elution peaks even at high loadings. Their great drawback is their softness, which limits the solvent flow-rates to about 1 cm/min and limits the length of the column to some 30 cm.

The solutes are bound to the resin by non-polar, hydrophobic interaction with the resin polymer matrix and the primary, perhaps the only function of the ionic

groups is to be hydrated and to cause the resin to be swollen and permeable. The hydrophobic interactions can be exploited in styrene–divinylbenzene polymers with no ionic groups, provided they have a suitably porous structure. Macroporous polymers like the Amberlite XAD resins (Rohm & Haas, Philadelphia, Pa., U.S.A.) have been used for liquid chromatography of polar organic compounds, including weak acids[9,10]. They absorb the non-ionic, protonated acids, and to a slight, but measurable extent they also absorb sodium salts of these acids. For chromatographic use, commercial resin beads are ground and screened, but the large and irregular particles of the ground resins cause the theoretical plate heights to be unduly large. They do, however, have the advantage of rigidity.

Macroporous polymers specially prepared for liquid chromatography, with particle diameters of 10 μm and less, are now produced commercially in Japan[11,12]. In this report we compare their performance with that of cation-exchange resins having comparable particle diameters.

EXPERIMENTAL

Apparatus

Liquid chromatography pumps of various kinds were used, principally the Model 6000 pumps from Waters Assoc. (Milford, Mass., U.S.A.), with ultraviolet absorbance detectors from Spectra-Physics (Santa Clara, Calif., U.S.A.). Columns were either of glass (Glenco, Houston, Texas, U.S.A.; high-pressure model), 6.3 mm I.D., or of stainless steel, 4 mm I.D. All columns were water-jacketed and maintained at a constant temperature of 55°.

Materials

Ion-exchange resins. Aminex 50W-X4, 20–30 μm, was obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.). Other resins of 10–15 μm diameter and 4% or 7% cross-linking were supplied by Hamilton (Reno, Nev., U.S.A.). All were cation exchangers with sulfonic acid groups on a styrene–divinylbenzene matrix.

Porous polymer gel. This was a styrene–divinylbenzene copolymer of macroporous structure without ionic groups, particle diameters 5 and 10 μm made by Toyo Soda (Tokyo, Japan).

Chemicals. High-quality products from various suppliers were used, and were recrystallized when necessary. Salicylamide, whose behaviour was studied in some detail, was recrystallized from water.

Solvent. Mixtures of ethyl alcohol with water were used. Commercial 95% alcohol was used, as it had excellent spectral purity. By "25% alcohol" we mean a mixture of 25 parts of 95% alcohol with 75 parts of water by volume. The volume ratio was controlled as carefully as possible; however, the composition of the (nominally) 95% alcohol stock may have varied from one bottle to another. Tests described below showed that the net retention volume of caffeine fell by 5% if the alcohol concentration rose by 1% (that is, from 25% to 26% by volume).

Column packing

Ion-exchange resins were packed as slurries in 25% alcohol, allowing them to settle by gravity and then applying moderate pressure. The porous polymer gel

was packed as a slurry in the same solvent that would be used in the chromatographic runs, namely 25% alcohol. It was packed down-flow in a pulsing mode.

RESULTS AND DISCUSSION

pH measurements and ionization constants

Though the columns were run at 55°, pH measurements were made at room temperature. Portions of effluents were collected and compared with standard aqueous buffers, generally 0.05 *M* phthalate or phosphate. The pH values of these buffers change little with temperature[13]. A greater uncertainty comes from comparing pH measurements in aqueous buffers with those in effluents containing 25% ethanol; however, the studies in which an accurate knowledge of pH was most important were made with salicylamide and we determined the ionization constant of this acid by titrating it in 25% alcohol containing 0.10 *M* potassium chloride at 25° and 55°, again calibrating the electrodes with aqueous buffers. We measured the ionization constant of acetaminophen (*p*-hydroxyacetanilide) in the same way. The ionization constants of the acids used in our study are shown in Table I.

TABLE I

IONIZATION CONSTANTS OF ACIDS USED IN THIS STUDY (pK_a UNITS AT 25°, REF. 14)

Acid	pK_a
Salicylamide*	8.45 (25°), 8.46 (55°)
p-Hydroxyacetanilide*	9.9
Benzoic acid	4.2
Caffeic acid	4.7
Cinnamic acid	4.45
p-Aminobenzoic acid	2.4 (pK_1), 4.9 (pK_2)
Nicotinic acid	4.8 (pK_1), 12.0 (pK_2)
Salicylic acid	2.9 (pK_1), 12.4 (pK_2)
Xanthine	7.7

* Our values.

Analgesic drugs on 4% cross-linked resin

These data were obtained on a column of Aminex 50W-X4 cation-exchange resin with eluents containing 25% ethyl alcohol. All were 0.10 *M* in Na^+ or NH_4^+ and were buffered with formic, acetic, phosphoric, citric or boric acid. The nature of the anion did not affect the retention, and the ionic strength had little effect.

Fig. 1 shows elution curves at two pH values, and Fig. 2 shows the effect of pH on retention volume for sodium and ammonium buffers. The pH has no effect on the elution of non-ionized compounds like caffeine and phenacetin, but with weak acids, the elution volume falls as the pH rises and becomes equal to the void volume at high pH. The uncharged acid molecules are retained, but the anions are not. In such cases the pH of half retention, where the capacity factor is half of its maximum, should equal pK_a[9,10,15]. Salicylic acid is stronger than acetylsalicylic acid by about 0.5 pK_a, and this fact makes it possible to separate these two acids below pH 5.

The case of salicylamide is interesting. From Fig. 2, its pH of half retention

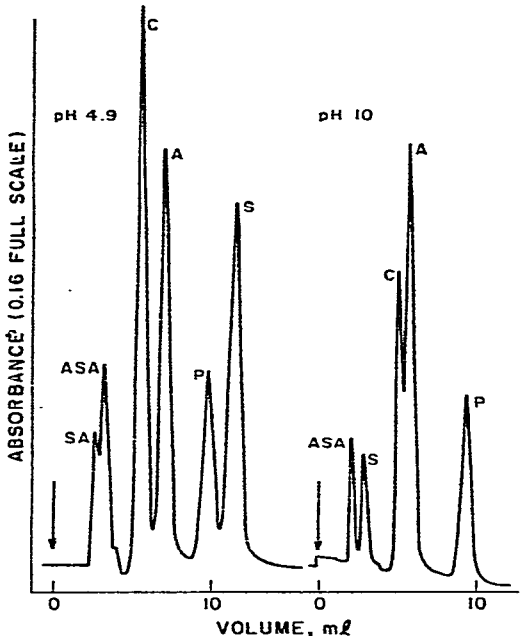


Fig. 1. Elution of analgesic drugs from the cation-exchange resin, Aminex 50W-X4-NH_4^+. Column, 20 × 0.63 cm; eluent, 25% alcohol, 0.1 *M* buffers (see text); temperature, 55°; flow-rate, 24 ml/h. SA = salicylic acid; ASA = acetylsalicylic acid; C = caffeine; A = *p*-hydroxyacetanilide; P = phenacetin (*p*-ethoxyacetanilide); S = salicylamide.

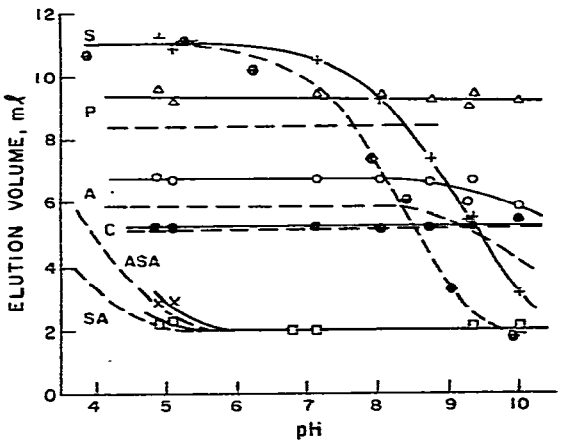


Fig. 2. Elution of analgesic drugs from Aminex 50W-X4; pH and counter-ion effects. Same column and conditions as Fig. 1. Dashed lines, Na^+ resin and buffers; solid lines, NH_4^+ resin and buffers. Abbreviations as in Fig. 1.

on the sodium-form resin was 8.2, which is somewhat less than the measured pK_a value. With ammonium counter-ions, however, the half-retention pH was one unit higher. The same conclusion was reached from a separate set of experiments (Fig. 3) with a different column of the same resin, using ammonia–ammonium nitrate

buffers. Potassium-loaded resin gave the same elution volumes as sodium-loaded, but the half-retention pH values were 8.1 for Na^+ and K^+, 9.05 for NH_4^+. The maximum retention (below pH 6) was the same for all three counter-ions, and the minimum in each case was zero, that is, the compound eluted at the void volume. In the intermediate pH range (8–9) the retention was very slightly greater in 0.1 *M* ammonium salt than in 0.02 *M*, namely, 0.1 ml out of 4–5 ml.

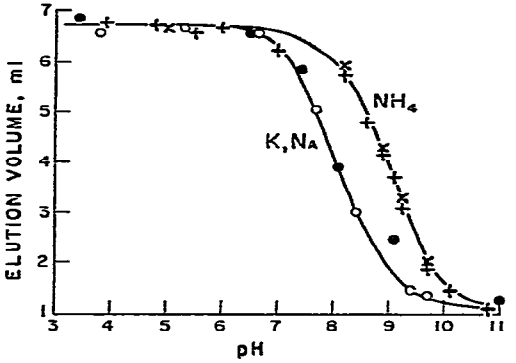


Fig. 3. Elution of salicylamide from Aminex 50W-X4; pH and counter-ion effects. Column, 12.5 × 0.63 cm; temperature, 55°; flow-rate, 24 ml/h; solvent, 25% alcohol. Counter-ions: ○, Na^+, 0.10 *M*; ●, K^+, 0.10 *M*; +, NH_4^+, 0.02 *M*; ×, NH_4^+, 0.10 *M*.

The reason for this behavior undoubtedly involves the acid nature of the ammonium ion. If stable $NH_4^+X^-$ ion-pairs were formed, one would expect significant retention at high pH, and this is not observed. The micellar fluid, that is, the solution within the swollen resin beads, has a high NH_4^+ concentration and a very low OH^- concentration because of Donnan exclusion. It must therefore have a relatively low pH, compared with the external solution, and the proportion of uncharged salicylamide, HX, to anions X^- must be relatively high.

There are signs of a similar behavior with the drug acetaminophen (*p*-hydroxyacetanilide), which we found to be a weak acid ($pK_a = 9.9$). The data of Fig. 2 do not extend to a high enough pH to determine its pH of half-retention in ammonium-form resin, but obviously it is higher than that in sodium-form resin.

In the low pH range, ammonium ions do not seem to have this effect. Tests performed with benzoic and cinnamic acids indicated the pH of half retention was about the same with sodium, potassium and ammonium counter-ions. For cinnamic acid this pH was 4.6.

The counter-ion effect was studied in detail with all the solutes shown in Fig. 2, as well as with chlorinated biphenyls[3]. Counter-ions used were Na^+, K^+, NH_4^+, and Ca^{2+}. The effects on retention were not great, and for some solutes, notably caffeine, they were almost nil. Retention increased in the sequence Na^+, NH_4^+, K^+, Ca^{2+} (except for the special effect of NH_4^+ at high pH, mentioned above). This was the inverse order of the bed volumes. Calcium-loaded resins showed the least swelling and the retention effect may simply be correlated with the lower water content and greater hydrophobic character of the calcium resins. For salicylamide and phenacetin the theoretical plate heights increased considerably when Ca^{2+} was substituted for

NH_4^+ or Na^+, a natural consequence of decreased swelling and retarded diffusion.

Fig. 4 shows the effect of pH on the retention of several compounds on Aminex 50W-X4-Na and Fig. 5 shows a chromatogram obtained with the Hamilton 4% cross-linked resin. The plate number for phenacetin in Fig. 5 is 3500 for a 24-cm column. The Hamilton 7% cross-linked resin gave a similar chromatogram with somewhat broader bands, showing better resolution of caffeic acid and xanthine. Salicylamide eluted before *p*-aminobenzoic acid.

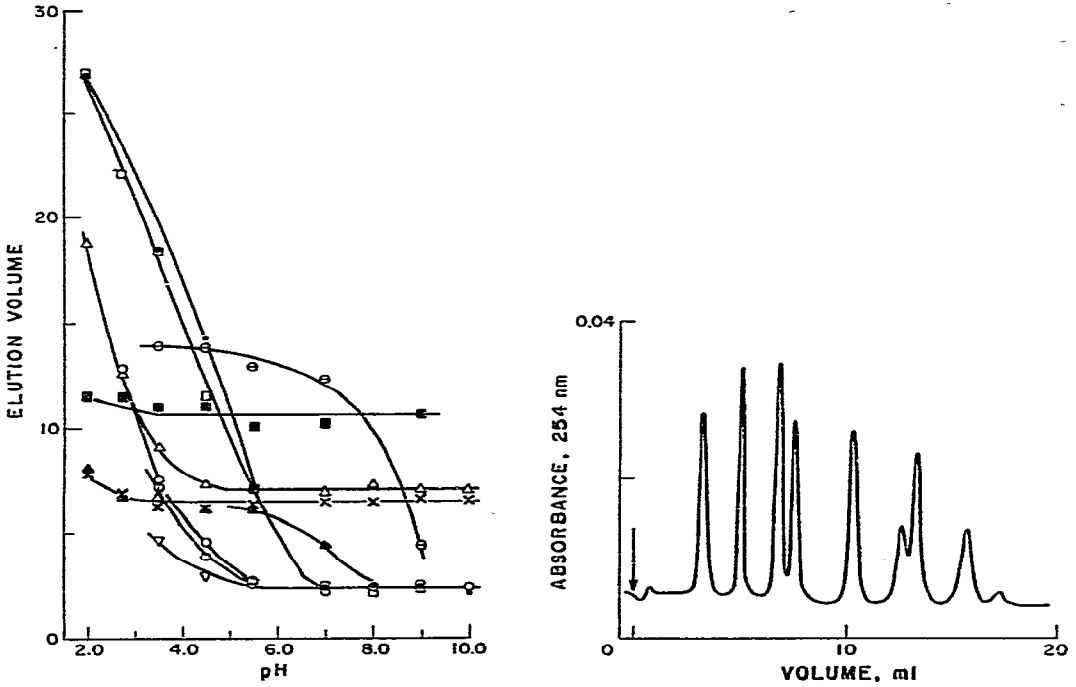


Fig. 4. Elution from Aminex 50W-X4-Na; pH effect. Column, 22 × 0.63 cm; temperature, 55°; solvent, 25% alcohol with 0.1 *M* Na^+, variable phosphate. Solutes: ●, caffeic acid; ○, nicotinic acid; □, *p*-aminobenzoic acid; ⊖, salicylamide; ■, phenacetin; △, trigonellin, ×, caffeine; ▲, xanthine; ⓘ, acetylsalicylic acid; ▽, salicylic acid.

Fig. 5. Elution from Hamilton 4% cross-linked resin, Na^+, 10–15 μm. 0.1 *M* Na^+, phosphate at pH 4.5 in 25% alcohol at 55°; flow-rate, 12 ml/h. Column, 24 × 0.63 cm. Peaks, in order of appearance, are: salicylic acid, nicotinic acid, xanthine plus caffeine, trigonellin, phenacetin, *p*-aminobenzoic acid, salicylamide, cinnamic acid, small unknown peak. Quantities injected were between 0.1 and 2.0 μg. Plate number was 3500 for phenacetin, 5000 for salicylamide.

Fig. 4 shows the effect of protonation on the retention of trigonelline (N-methylpyridinium-3-carboxylate), which above pH 5 is a dipolar ion. At lower pH values the carboxylate ion is protonated, and the molecule has a net positive charge, which makes it absorb as a cation through ion exchange. The dipolar ion is absorbed also, but more weakly. The cases of *p*-aminobenzoic acid ($pK_1 = 2.4$, $pK_2 = 4.9$)[14] and nicotinic acid ($pK_1 = 4.9$, $pK_2 = 12$) are similar. It appears that the uncharged form of *p*-aminobenzoic acid is retained with k' of about 8, while the uncharged form of nicotinic acid is hardly retained at all.

Figs. 6 and 7 show the effect of pH on retention by the 7% cross-linked resin in the presence of sodium sulfate. The buffering ions are phosphate, and the total sodium-ion concentrations range from 0.25 to 0.30 *M*. Extensive experiments showed that the retention was the same in the presence as in the absence of sodium sulfate; 0.005 *M* phosphate gave the same retention as 0.05 *M* phosphate. Retention is, therefore, insensitive to ionic strength. Figs. 6 and 7 cover a wider pH range than Fig. 4, and show that the pH of half-retention for the uncharged, monoprotic acids, benzoic, cinnamic, caffeic, and xanthine are very close to their pK_a values. There is no evidence of ion-pair formation.

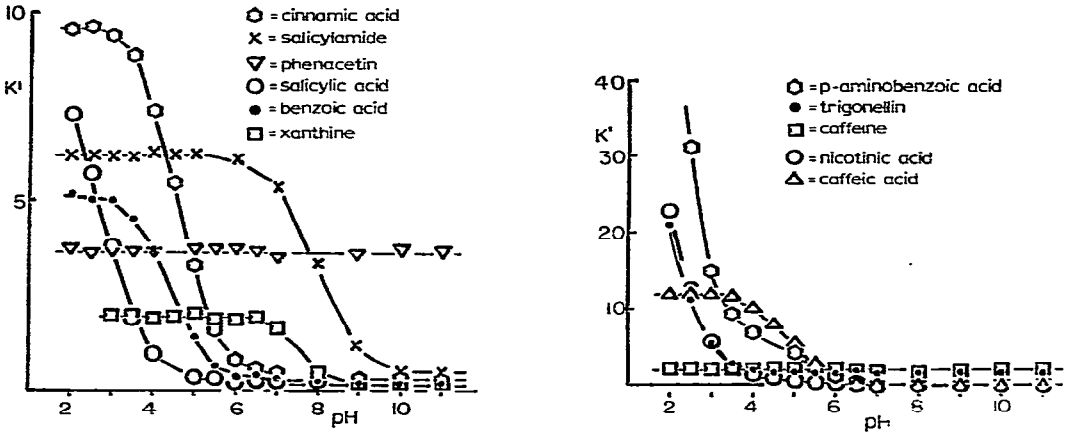


Fig. 6. Elution from Hamilton 7% cross-linked resin, Na^+; effect of pH. 25% alcohol, 0.1 *M* in Na_2SO_4 and 0.05 *M* in Na_2HPO_4; pH adjusted by adding NaOH or H_3PO_4; temperature, 55°, flow-rate 16 ml/h.

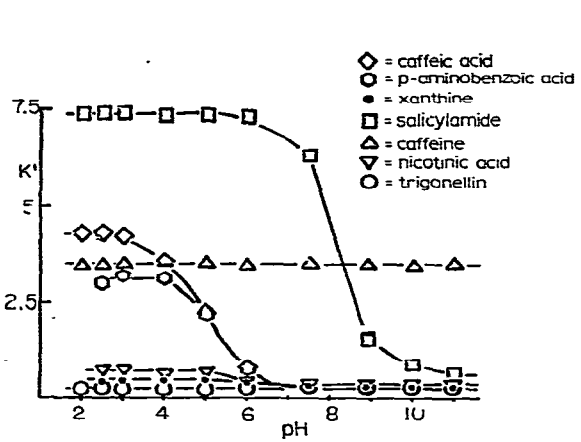
Fig. 7. Elution from Hamilton 7% cross-linked resin, Na^+; effect of pH. Same column, flow conditions, buffers and temperature as in Fig. 6.

Tests were also made in the presence of 0.017 *M* sodium dodecyl sulfate. This compound quadrupled the retention of nicotinic acid and trigonelline at pH 3, and almost doubled the retention of *p*-aminobenzoic acid, while having little or no effect on the other compounds. Evidently it forms ion pairs with the cationic forms of the three compounds that it affects.

Studies with porous polymer gel

Figs. 8 and 9 show the retention of two groups of compounds on the macroporous non-ionic polymer, TSK-LS110. The same pH dependence is found as was found with the cation-exchange resin, but there is a striking difference between the strengths of retention of the compounds shown in Figs. 8 and 9. Cinnamic and salicylic acids are held much more strongly on the porous polymer than on the ion-exchange resins. Phenacetin is held twice as strongly as caffeine on the ion-exchange resins, but 7–8 times as strongly on the porous polymer. On the other hand, trigonelline and nicotinic acid, which exist as dipolar ions over large pH ranges, are hardly absorbed at all by the porous polymer, and *p*-aminobenzoic acid is absorbed only weakly. One would not expect dipolar ions to be strongly absorbed by a non-

polar, non-ionized medium of low dielectric constant such as polystyrene; however, significant sorption of *o*-aminobenzoic acid (anthranilic acid) on octadecyl-coated silica has been observed[15]. The caffeine–phenacetin retention ratios suggest that the porous polymer gel has a preference for benzene-ring aromatic compounds, as opposed to heterocyclic structures like xanthines.



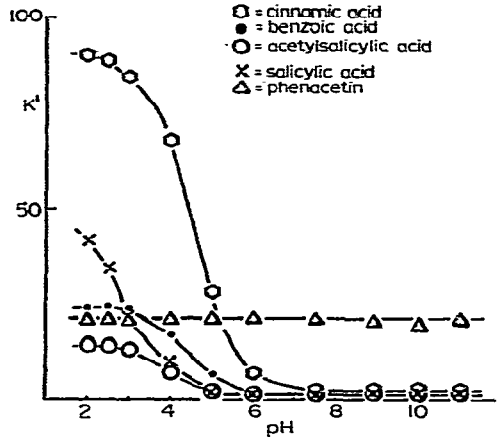


Fig. 8. Elution from porous polystyrene gel. Column, 25 × 0.4 cm; same buffers, flow-rate and temperature as in Fig. 6.

Fig. 9. Elution from porous polystyrene gel. Same column and conditions as in Fig. 8.

Retention of acidic compounds drops to zero at high pH. Under the conditions of Figs. 8 and 9 there is no sign of anion absorption or ion-pair formation, in contrast to the observations of Pietrzyk and Chi-Hong Chu[10].

Ion pairing in porous polymer gel

To examine the role of ion-pairs, tests were made with eluents containing sodium dodecyl sulfate (0.017 *M*) and tetrabutylammonium ions (0.020 *M*). Tetrabutylammonium ions (TBA) were added as the hydroxide, and the pH was adjusted to the desired value by adding concentrated phosphoric acid. Sodium dodecyl sulfate increased the retention of cationic species at low pH, as expected. The behaviour of TBA is shown in Fig. 10.

Without added TBA, salicylamide is not retained at high pH, but emerges at the void volume. The retention rises to a maximum as the pH falls and the compound assumes its uncharged form. The pH of half retention is 8.1, the same value found with sodium-form ion-exchange resin (Figs. 2 and 3). Theoretically the pH of half retention should equal pK_a. We found pK_a to be 8.4 (see above). The discrepancy is small and may or may not be significant.

In the presence of TBA, salicylamide is retained at high pH, presumably as an ion-pair, and k' is about half that of uncharged salicylamide. Retention is halfway between the high and low pH values at pH 8.3. Theoretically this should occur at pH = pK_a (eqn. 4 in ref. 10).

Nicotinic acid is affected by TBA in an unexpected way. In the absence of

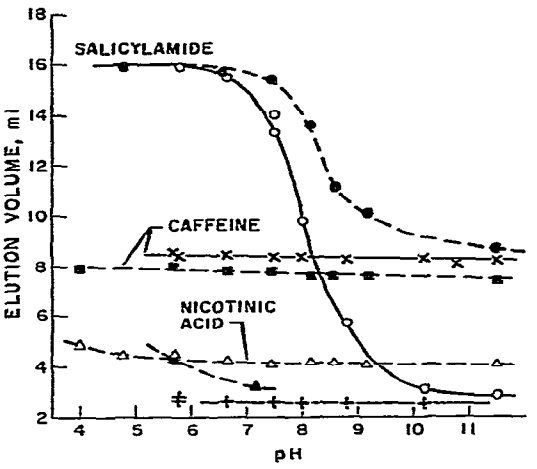


Fig. 10. Elution from porous polystyrene gel; effect of ion pairing. Same column as in Fig. 8; solvent, 25% alcohol, 0.10 *M* in Na_2HPO_4; flow-rate, 30 ml/h; temperature, 55°. ———, without ion-pairing reagent; — — —, eluent 0.020 *M* in $N(C_4H_9)^+$ (TBA). Phenacetin (not shown) eluted at 38.0 ml, independent of pH or TBA; trigonellin (not shown) always eluted at the void volume, 2.4–2.5 ml. ○ and ●, salicylamide; × and ■, caffeine; + and △, nicotinic acid; ▲, *p*-aminobenzoic acid.

TBA it is not retained, but emerges at the void volume. It exists as a neutral species, probably the dipolar ion $C_5H_4NH^+COO^-$, over the whole pH range shown in Fig. 10. With TBA present it is retained with $k' = 0.6$. Similar behaviour is shown by *p*-aminobenzoic acid, in which the dipolar ion predominates between pH 2.4 and 4.9. It is not clear how TBA can promote the sorption of a dipolar ion. Trigonelline, which exists as a dipolar ion over the pH range of Fig. 10, is unretained whether TBA is present or not.

The retention of caffeine is reduced slightly by TBA, that of phenacetin is unaffected.

Plate heights and resolution

For the conditions of Figs. 8 and 9 with linear velocity 2.4 cm/min, the plate heights for an unretained solute (xanthine) was less than 0.1 mm, whereas the plate height for caffeine was 0.2 mm with some tailing. Plate heights are not as good as those found with the ion-exchange resins; however, the resolution is generally better because of larger differences in retention. To exploit these retention differences one must use a gradient. Fig. 11 shows the separation of 12 compounds on a column of TSK gel, using a convex pH gradient with rapid composition change at first and slower change later. The alcohol concentration was constant, 25% by volume, and the pH of the phosphate buffer changed from 2.5 to 8.5 over 2 h.

With an alcohol concentration gradient from 25% to 95% we found that the column packing was damaged. It seemed that the gel particles expanded and then, when the solvent was changed back to its original composition, the particles did not rearrange themselves to their original distribution; the quality of packing, tested by the width of an unretained peak, was now very poor. The gel itself was not irreversibly spoiled, as the column could be emptied and repacked to give good performance

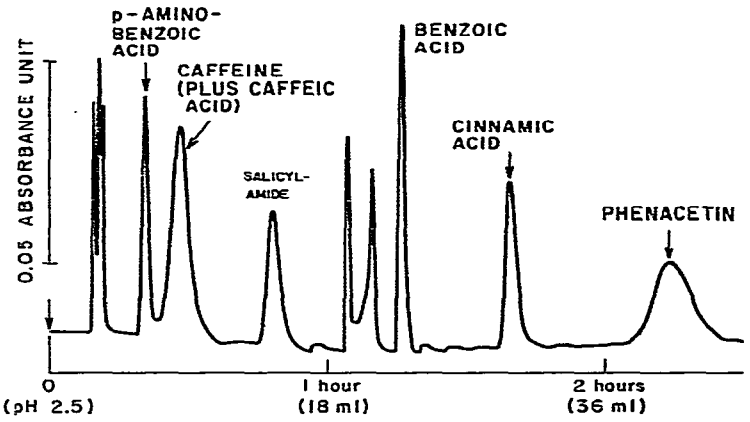


Fig. 11. Separation of polar aromatic compounds on porous polystyrene gel. Same column as in Fig. 8; flow-rate, 18 ml/h; pressure, 70 bar; eluent, phosphoric acid (0.2 *M*) to Na_2HPO_4 (0.2 *M*) over 2 h; gradient No. 3 on Waters Assoc. programmer (see text). Peaks in order of appearance are: trigonellin, xanthine, nicotinic acid (close together); *p*-aminobenzoic acid; caffeic acid and caffeine (together); salicylamide, acetylsalicylic acid, salicylic acid, benzoic acid, cinnamic acid, phenacetin. Quantities injected were 0.5–7.5 μg.

again but it is evident that the use of solvent gradients is limited with this packing. Perhaps one would get better performance in a wider column.

Effect of solvent composition

The effect of alcohol concentration on retention was measured for phenacetin and caffeine on the porous polymer gel and on the Hamilton 7% cross-linked resin, from 20% to 30% of commercial (95%) alcohol by volume, that is, from 19% to 28.5% of ethanol. In all cases, straight-line plots of $\log k'$ against volume fraction of alcohol were obtained. The retention of phenacetin fell by a factor of 2.2 on the polymer, 2.1 on the ion-exchange resin, over the interval named; that of caffeine fell by a factor of 1.65 on both sorbents. The linear relation between the free energy of sorption and the volume fraction of alcohol in the solvent has been noted by several workers, including Karger *et al.*[16], who have made a study of the hydrophobic effect in liquid chromatography.

CONCLUSION

Most liquid chromatography today is "reversed-phase", in which the stationary phase is non-polar and hydrocarbon-like. The most popular material is porous silica coated with octadecyl groups. Horváth and co-workers[15,17] have used solubility theory and the theory of solvophobic interactions to predict distribution ratios of molecules that have a hydrocarbon, or hydrophobic, part and a polar, or hydrophilic, part. Among the solutes they studied experimentally were benzoic and cinnamic acids. The effect of pH on the distribution of weak organic acids, bases and ampholytes was studied experimentally and theoretically[15], using octadecyl–silica as the stationary phase.

The drawback to octadecylsilica is that it can only be used in a limited pH

range, at most 2–8. Porous polymer gels can be used over the entire pH range, as can ion-exchange resins. Non-ionic porous polymer gels are easier to interpret than ion-exchanging polymers; they show greater differences in distribution ratios between different solutes, and they can be used in columns with fairly high pressure gradients. Ion-exchange resins with low cross-linking allow faster mass transfer than porous polymers because of their looser internal structure, and therefore they give smaller theoretical plate heights. Ionic attractions and repulsions are superimposed on solvophobic interactions, and therefore the retention of ionogenic solutes depends on pH in a different way. In certain cases the counter-ion affects the retention. Ion-exchange resins and non-ionic porous polymers can thus supplement each other in solving analytical problems. The disadvantage of ion-exchange resins is their softness.

Styrene–divinylbenzene porous polymer gels have recently been used for chromatography of fatty acids[18], aromatic hydrocarbons and benzoate esters[19].

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