Journal of Chromatography, 180 (1979) 145–156

CHROM. 12,256

CHROMATOGRAPHY OF XANTHINES ON ION-EXCHANGE RESINS

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(Received May 7th, 1979)

SUMMARY

Short columns of a 4% crosslinked cation-exchange resin gave good chromatography of xanthines, including caffeine, theophylline and hypoxanthine, and related polar aromatic compounds. Elution volumes and sequences can be modified by changing pH, solvent composition and resin counter-ion. A macroporous cation-exchange resin showed exaggerated counter-ion effects. A method is described for determining caffeine and theophylline in blood serum, using the 4% crosslinked resin with aqueous sodium phosphate eluent of pH 7.5; the temperature was 65°. Detection limits are 10 ng and less.

INTRODUCTION

It is well known that ion-exchange resins absorb non-ionized organic compounds, particularly those having aromatic character, and that they can be used as stationary phases for the chromatography of these compounds. Mass transfer is slow in conventional resins, compared to bonded-phases, but resins of small particle size and low crosslinking give satisfactory resolution in a reasonable time and have the advantages of easy packing, high capacity and stability over a wide pH range. Cation-exchange resins absorb uncharged acids and exclude their anions; thus, retention can be modified by adjusting the pH. "Ion-exclusion chromatography" uses this property[1]. The relation of pH to the retention of weak acids and bases by non-polar bonded-phases and porous polymers has been studied by Horváth *et al.*[2], Pietrzyk and Chu[3] and ourselves[4]. Ion-exchange resins are somewhat simpler to treat than non-ionic absorbents in the sense that co-ions are completely excluded.

Retention of organic solutes by ion-exchange resins can be controlled by choosing pH, solvent composition and the nature of the counter-ion. To explore the effect of these factors we have measured retention volumes of a series of xanthines and related polar aromatic compounds that exemplify certain structural types. Two resins were used, a conventional or "gel-type" cation-exchange resin with 4% crosslinking and a macroporous sulfonated polystyrene cation exchanger.

Besides studying chromatographic parameters, we have used the gel-type resin to determine caffeine and theophylline in blood serum. Many publications have appeared recently on the measurement of theophylline in serum by liquid chroma-

tography. The reason for this interest is that theophylline is used as a drug to treat asthma, and its therapeutic range is narrow. Below 10 mg/l in blood it is ineffective, and above 20 mg/l it may have bad side effects. Most published methods for its measurement use reversed-phase C_{18} silica bonded packings[5–9]. Some use normal-phase chromatography on silica[10,11]; theophylline must then be extracted by a solvent, but the extraction reduces interferences. Anion-exchange resins have been used[12] and so has a pellicular cation exchanger[13]. A cation-exchange resin was used to determine caffeine in coffee[14].

It is probably superfluous to propose yet another method to measure theophylline and caffeine in blood and coffee, but as we have noted, ion-exchange resins have certain advantages, and our method is simple and effective.

EXPERIMENTAL

Equipment

Waters Model 6000-A pumps and a Milton Roy Minipump were used, with a single-wavelength, 254-nm ultraviolet detector (Chromatronix) or a variable-wavelength detector (Schoeffel Model SF-770). Samples were introduced by syringe and septum or by a Rheodyne Model 7120 sample-injection valve. Two kinds of columns were used: Glenco Model 3202 high-pressure glass columns, 6.3 mm I.D., and stainless-steel columns, 4.6 mm I.D. and 10–15 cm long. All columns were water-jacketed and maintained at 65° by circulating water baths.

Materials

Resins. The "gel-type resin" was Aminex 50W-X4 (Bio-Rad Labs., Richmond, Calif., U.S.A.), 20–30 μm diameter, a sulfonated polystyrene with 4% divinylbenzene; the "macroporous resin" was Hitachi 3011-S, a sulfonated polystyrene with particle diameter 10–20 μm.

Buffers, solvents. Most experiments were made with phosphate buffers, 0.10 *M* in sodium or other singly-charged cation, 0.05 *M* in doubly-charged cations. Acetate buffers were used for most of the work with calcium and magnesium ions and for some of the tests reported in Tables I and II. All buffers were filtered and degassed

TABLE I

EFFECT OF COUNTER-ION ON RETENTION: GEL-TYPE RESIN

Aqueous solutions, pH 5–6. Corrected retention volumes in ml for a particular column are quoted.

Compound	*Counter-ion*						
	Li	*Na*	*K*	NH_4	$N(CH_3)_4$	*Mg*	*Ca*
Caffeine	3.2	2.45	2.25	2.25	1.0	2.8	2.5
Theophylline	2.35	2.0	1.85	1.7	1.0	1.7	1.8
Hypoxanthine	2.0	1.7	1.55	1.5	1.2	1.8	1.55
Trigonelline	1.7	1.05	0.8	1.0	0.55	1.7	1.8
Salicylamide	5.4	5.2	5.2	5.0	6.6	—	5.0
Phenacetin	7.5	5.9	5.5	6.3	5.5	—	6.4
Tyrosine	2.8	3.1	—	—	—	3.3	3.1

TABLE II

EFFECT OF COUNTER-ION ON RETENTION: MACROPOROUS RESIN

Aqueous solutions, pH 5–6. Corrected retention volumes in ml for a particular column are quoted.

Compound	*Counter-ion*						
	Li	*Na*	*K*	NH_4	$N(CH_3)_4$	*Mg*	*Ca*
Caffeine	2.8	2.0	1.55	1.6	0.35	1.75	2.7
Theophylline	2.5	2.0	1.85	1.5	0.45	1.35	2.65
Hypoxanthine	3.1	3.25	2.4	1.75	0.9	2.15	3.1
Trigonelline	1.8	—	—	—	—	1.95	3.0
Salicylamide	9.6	—	—	—	3.3	—	14.5
Phenacetin	6.0	6.4	—	5.4	1.3	5.8	10.2

before use. Ethanol (95%) was laboratory grade; acetonitrile was of chromatographic grade from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.).

Chemicals. These were obtained from various sources; theophylline, hypoxanthine and salicylamide were recrystallized. The compound diethylcaffeine (1,7-diethyl-3-methylxanthine) was prepared in our laboratory by Mr. Kenneth Kolonko, as follows.

A 10-g amount of 3-methylxanthine (Aldrich, Milwaukee, Wisc., U.S.A.) was slurried in 75 ml warm absolute ethanol, and 10 g potassium hydroxide in 100 ml absolute ethanol were added. The mixture was heated to boiling, and enough water added to dissolve the sparingly-soluble salt. It was cooled to 40° and a solution of 20 g ethyl iodide in 10 ml ethanol was added; then the solution was refluxed for 6 h and left overnight. Next day, a second quantity of ethyl iodide in ethanol, as before, was added and the mixture was again refluxed for 6 h; after standing overnight, the solution was evaporated to dryness in a rotary evaporator. The residue was dissolved in water and extracted with three portions of chloroform. The extracts were combined, dried and evaporated; the solid remaining was sublimed at 160° and 1 mm pressure to yield 4.9 g (a 37% yield) of diethylcaffeine.

Column packing

The glass column was packed, with the lower end-fitting in place, by pouring in the resin slurry, attaching the upper end-fitting and pumping buffer until the resin had settled; then the upper end-fitting was removed and more resin slurry added, repeating the pumping and addition until the column was sufficiently packed. To pack the steel tubes, the bottom end-fitting was attached and the other end of the tube was connected to a short length of tube of the same diameter, then through a reducing union to a wider tube that served as a reservoir for the resin slurry. The pump was attached, and the slurry pumped from the reservoir into the column at a speed equal to the flow-rate that would be used in the experiments; for the gel-type resin this was 0.3 ml/min. If this rate was greatly exceeded the resin packed down and plugged the column. Pumping at 0.3 ml/min was continued for some 30 min until the back pressure became constant (at about 200 p.s.i. = 15 bar). The pump was stopped, the column disconnected and closed with the upper end-fitting.

Void volume measurement

With the column in place, the dead volume (equal to the true void volume plus the volume of the connections) was found by injecting a non-retained substance and measuring the distance on the chart paper from the point of injection to the top of the peak. The most suitable non-retained substance was sulfosalicylic acid. Uric acid could be used at pH values above 6.

In measuring retention volumes, the rate of flow from the pump was always checked by collecting the effluent in a graduated cylinder over a measured time.

Procedure for blood serum

Because the analysis of blood serum was supplementary to the main object of this research, experimental details are given below under Results and Discussion.

RESULTS AND DISCUSSION

Chromatograms of standard mixtures

To assess column performance a Glenco glass column was packed with Aminex 50W-X4 resin as described above, and a few trials were made to find suitable conditions for the separation of theophylline, theobromine, caffeine and diethylcaffeine. A chromatogram with 0.05 *M* sodium phosphate in 10% ethanol at pH 7.8 is shown in Fig. 1. The peaks are well spaced, there is baseline separations, and the plate number

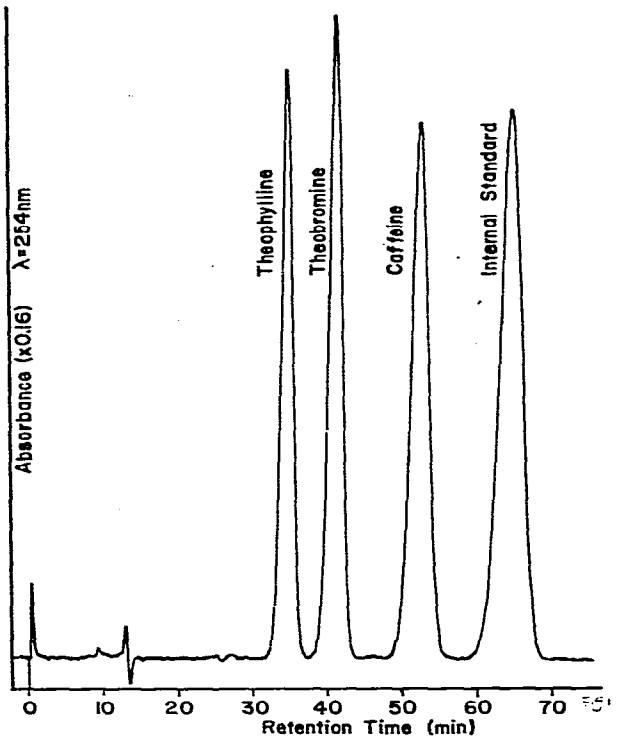


Fig. 1. Chromatogram of pure compounds on gel-type resin. Internal standard is diethylcaffeine. Column, 21 cm × 6.3 mm; flow-rate, 0.1 ml/min; temperature, 65°; eluent, sodium phosphate buffer, 0.05 *M* in phosphate, in 10% ethanol, pH 7.8.

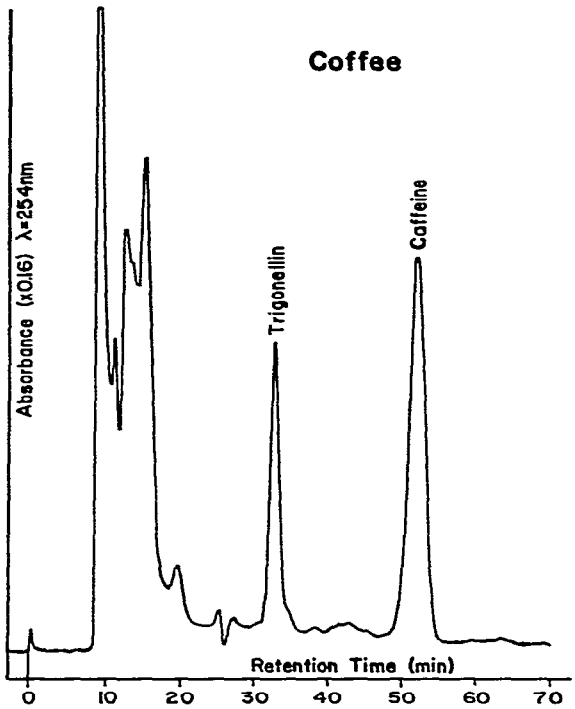


Fig. 2. Chromatogram of beverage coffee; sample size, 2 μl. Conditions as in Fig. 1.

for caffeine is 2200. A chromatogram of coffee in the same column appears in Fig. 2.

Elution times are long, $\geqslant$1 h. Tests were therefore made with a shorter column and a faster flow. Figs. 3 and 4 show the performance of a column 10 cm $\times$ 0.46 mm I.D. packed with the same resin, at flow-rate 0.3 ml/min (1.5 cm/min linear rate). Analysis time is now 10 min, and resolution is still good. The plate number for caffeine is 900.

The mass of caffeine in Fig. 3b is 150 ng, and it would have been easy to detect 5 ng. An advantage of small columns and low retention volumes is the sensitivity that comes from small peak volumes.

Theophylline and hypoxanthine are imperfectly resolved (Fig. 3a), and one asks whether resolution could not be improved at the expense of speed by using a longer column at the same linear flow-rate. The problem with soft resins is that longer columns pack down more easily than short ones. It is not the pressure gradient or flow-rate that decides whether the resin will collapse and plug the column, so much as the total pressure across the column. We performed many runs with a column 15 cm $\times$ 0.46 cm I.D., but the plate numbers for caffeine seldom exceeded 1100.

Effect of pH

Theophylline and hypoxanthine are weak acids. We measured their ionization constants by glass-electrode titration in 0.1 *M* potassium chloride in water and aqueous ethanol at two temperatures. Our values for pK_a were: (1) Theophylline in water 8.65 (25°), 7.95 (60°); in 35% ethanol 8.4 (60°); (2) Hypoxanthine in water 8.7 (25°); in 35% ethanol 8.7 (60°). We expected that the non-ionized forms of these compounds

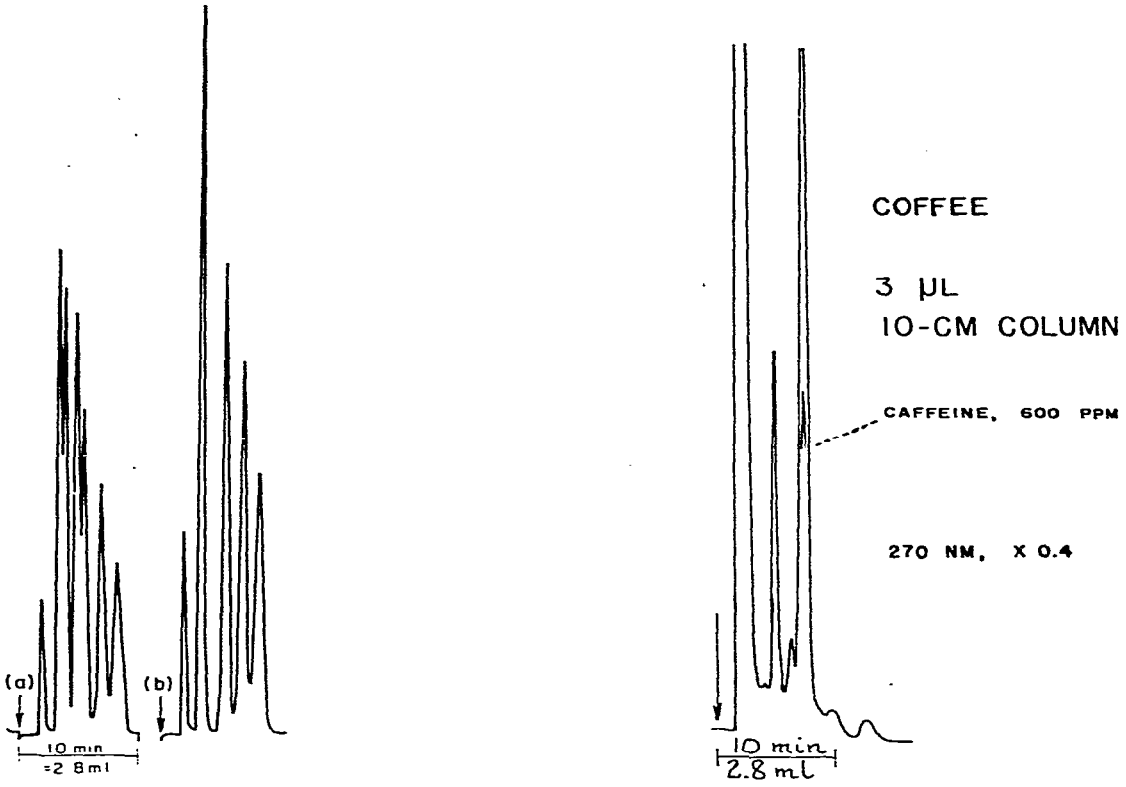


Fig. 3. Chromatogram of pure compounds on gel-type resin. Column, 10 cm × 4.6 mm; flow-rate, 0.28 ml/min; temperature, 65°; eluent as in Fig. 1 with pH = 8.3. Detection at 270 nm, full-scale absorbance 0.1. Curve (a), peaks in order of appearance are uric acid, theophylline, hypoxanthine, trigonelline, theobromine, caffeine, diethylcaffeine. Curve (b), like (a) with hypoxanthine and trigonelline absent. Quantity of caffeine in (b) = 150 ng.

Fig. 4. Chromatogram of coffee; column and conditions as in Fig. 3. Quantity of caffeine, 1.8 µg.

would be retained by the cation-exchange resin while their anions would not be, and this is what we found. Fig. 5 shows the capacity factors in phosphate buffers with first sodium, then potassium counter-ions. The solvent was 10% ethanol, the temperature was 65°, and all measurements were made with the same resin in the same column. The solid curve is the calculated curve for theophylline, taking $k' = 2.5$ at low pH, zero at high pH, and $pX_a = 8.25$ (ref. 3).

The column temperature was 65°, while the pH values were measured in both influent and effluent at room temperature. The tables presented by Bates[15] show, however, that the pH of a standard phosphate buffer decreases by only 0.03 unit in going from 25° to 60°. The pH of an ammonia–ammonium ion buffer, on the other hand, drops by 1.2 unit in the same temperature range. This is the reason for the apparent shift of the pH of half retention that we reported for salicylamide when ammonium ions were substituted for sodium ions in the resin and the flowing buffer[4]. Our "ammonium-ion effect" was spurious.

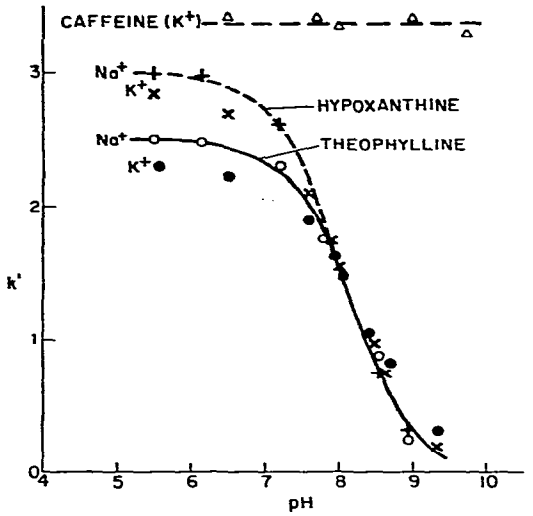


Fig. 5. Effect of pH on retention. Column, 15 cm × 4.6 mm; flow-rate, 0.3 ml/min; temperature, 65°; Eluents, buffers 0.10 *M* in Na^+ or K^+ with phosphate to give desired pH, 10% ethanol. Continuous line is a calculated curve; see text.

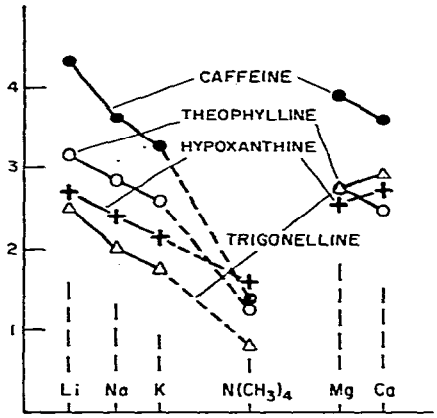
Effect of counter-ion

Fig. 5 shows a small but definite difference in retention between the sodium and potassium forms of the resin. A series of tests was therefore made in which a 15-cm column of gel-type resin was converted from one ionic form to another, without opening the column or adding or subtracting resin. The retention of a series of model compounds was measured at two pH values, about 5 and 6, with each ionic form, using aqueous acetate buffers. In this pH range the retentions should be independent of pH, and so they were. Again, all measurements were made with the column at 65°.

One of the compounds injected was always sulfosalicylic acid to check the void volume. The void volume depended on the ionic form, being least with the most swollen resins (Li and $N(CH_3)_4$; void volume 0.40 ml) and greatest with the least swollen resin (Ca; void volume 0.82 ml). The void volume was subtracted from the measured retention volumes to give the actual retention caused by sorption on the resin. The corrected volumes are listed in Tables I and II. Table II gives a comparable set of data found with the macroporous resin, Hitachi 3011-S, in various ionic forms.

Fig. 6 presents in graphical form the results of a second set of data from an independently-packed column of the gel-type resin. The data are not quite the same as those tabulated, but they are consistent. They point out one regularity that seems to exist: the dipolar ion, trigonelline, is more strongly held by divalent than by univalent counter-ions. We suggest that strong local electric fields exist in the divalent-ion resins because the stable position of the divalent cation is near *one* of the fixed ions, not between two of them; see Fig. 7. A strongly polar molecule like trigonelline would be held as shown.

We must then ask why the amino acid tyrosine, which exists as a dipolar ion in the pH region used[16], does not behave in the same way. Perhaps the π-electron overlap between the aromatic ring of tyrosine and the polystyrene resin matrix is



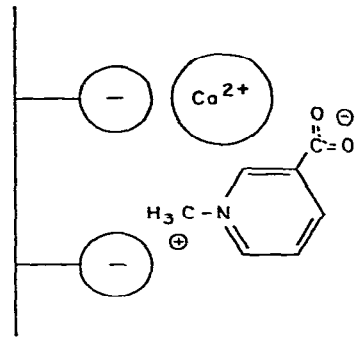


Fig. 6. Effect of counter-ion on retention.

Fig. 7. Model for retention of trigonelline on a calcium-loaded resin.

the dominant factor. We have, however, noted in earlier work[17,18], some of it unpublished, that calcium-form resins hold aromatic hydrocarbons more strongly than sodium-form resins.

The drop in retention along the counter-ion sequence Li–Na–K–NH_4–$N(CH_3)_4$ is very obvious, and it may be due to a combination of resin swelling, which makes the resin matrix more accessible to the solute molecules, and ionic size, which blocks access to the polymer matrix. The methyl groups of $N(CH_3)^+$, not having π-electrons, do not bind the aromatic solute molecules.

The blocking effect of $N(CH_3)^+$ is especially marked in the macroporous resin, where the aromatic solute molecules are probably adsorbed on the surface of the micro-particles of highly crosslinked polymer, rather than associating with the dispersed polymer chains of the gel resin.

Effect of solvent

Tables III and IV show the effect of the solvent modifiers, ethanol and acetonitrile. In Table III, ethanol lowers retention the most for the most hydrophobic compound, caffeine, and least for the most hydrophilic or polar compound, trigonelline. Theophylline, having two methyl side-chains, is more hydrophobic than hypoxanthine and more affected by ethanol. The correlation with hydrophobic

TABLE III

EFFECT OF SOLVENT ON RETENTION: GEL-TYPE RESIN, Na FORM

Different columns are compared, so capacity factors, k', are quoted; pH = 5–6.

Compound	k'		
	0% ethanol	*10% ethanol*	*20% ethanol*
Caffeine	5.4	3.5	2.7
Theophylline	4.2	3.0	2.5
Hypoxanthine	3.6	3.2	2.6
Trigonelline	2.7	2.75	2.6

TABLE IV

EFFECT OF SOLVENT ON RETENTION: GEL-TYPE RESIN, Ca FORM

Same column used throughout; corrected retention volumes are quoted in ml; pH = 5–6.

Compound	*Retention volumes*		
	Water	*CH_3CN (10%)*	*Ratio*
Diethylcaffeine	6.3	2.7	0.43
Caffeine	4.2	2.15	0.51
Theophylline	3.0	1.7	0.57
Theobromine	2.75	1.6	0.58
Hypoxanthine	2.75	1.9	0.69
Trigonelline	3.2	3.05	0.95
Acetaminophen	4.0	2.5	0.63
Phenacetin	11.0	5.4	0.49
Tyrosine	5.0	5.8	1.15

character is even more evident in Table IV. The hydrophilic dipolar ions, trigonelline and tyrosine, are retained as strongly in 10% acetonitrile as in water. One recalls that in solvent mixtures like these, the interior of the gel resin in predominantly aqueous[19,20].

Caffeine and theophylline in blood serum

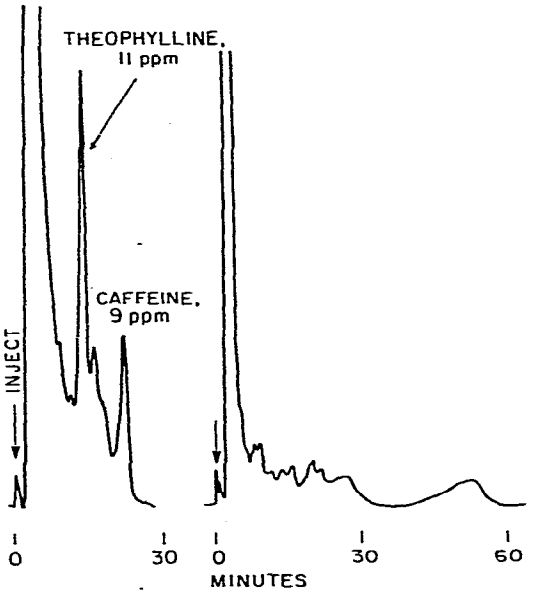
In seeking the best conditions for the chromatography of these compounds in serum or another matrix one must consider not only the separation of the caffeine and theophylline peaks from one another, but their separation from such other peaks as the matrix may produce. It is therefore an advantage to be able to move the peaks backwards or forwards by changing the parameters we have discussed. We decided to use aqueous buffers to avoid the solvent fronts that emerge when aqueous samples are injected into a mixed-solvent eluent, and to use sodium counter-ions, which generally give the sharpest peaks and the best plate numbers. A low pH was chosen to postpone the appearance of the theophylline peak until after most matrix peaks had emerged.

The method was tested with serum from a normal subject taken in the morning after overnight fasting. First a blank was taken, then, on another day, the subject breakfasted on a pot of coffee and 300 mg of theophylline and had blood drawn 1½ h later. The chromatograms of the two sera are shown in Fig. 8. Comparing the peak heights with those from a standard caffeine–theophylline solution, the serum was found to contain 11 mg/l of theophylline and 9 mg/l of caffeine. Obviously the test is sensitive enough to measure theophylline concentrations in the therapeutic range of 10–20 mg/l.

The serum was injected directly without pretreatment and without a guard column. After several injections, totalling 150 μl of serum, the back pressure showed that the column was becoming plugged; also, the large peak following the void volume became unduly broad. The column could be restored partially, but not completely, by pumping 0.1 *M* sodium hydroxide for 2 h.

The test sera were clear because the subject had fasted. After eating, the serum is cloudy and plugs the column sooner. We therefore tried precipitating the protein

with trichloracetic acid (TCA). A 100-μl volume of serum was mixed with an equal volume of 25% TCA and the mixture was centrifuged, then 10–20 μl of the supernatant was injected into the column. The result is shown in Fig. 9. At least 90% of the caffeine and theophylline is recovered after TCA treatment.



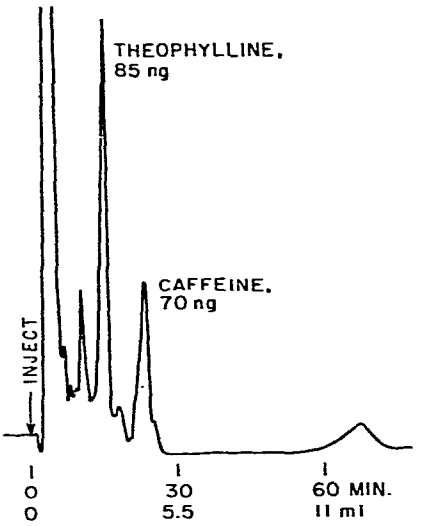


Fig. 8. Chromatograms of blood serum (injected directly; 5-μl samples). Column, 15 cm × 4.6 mm; packed with gel resin; flow-rate, 0.2 ml/min; temperature, 68°; eluent, aqueous 0.05 *M* Na_2HPO_4 with H_3PO_4 added to pH 7.5. Detection at 270 nm, 0.02 a.u.f.s.

Fig. 9. Chromatogram of serum deproteinized with TCA. Conditions and detection as Fig. 8.

Thanks to Prof. A. L. Dickinson of the Human Performance Laboratory of the University of Colorado, we were able to run 50 samples of serum taken from athletes who had ingested caffeine and were studying its effects on stomach emptying during exercise. These samples were treated with TCA as described, and 20-μl portions of the supernatant were injected into the 15-cm resin column. These samples showed many more peaks than the "clean" serum of Figs. 8 and 9. Two of the most complex chromatograms are shown in Fig. 10. Because of the baseline "noise", 1 mg/l of caffeine was about the limit of detection; however, 4–5 mg/l could easily be seen. Theophylline could have been detected in the therapeutic range, had it been present.

Our technique needs more development if it is to be offered as a clinical procedure for caffeine or theophylline, but there is little doubt that, knowing what we do about the chromatographic parameters ,this could be done. A guard column was used by other workers[9]; we could do the same. Calcium counter-ions might be substituted for sodium; they give somewhat larger plate heights, but the calcium resin is more rigid and could be packed into longer columns with faster flow-rates.

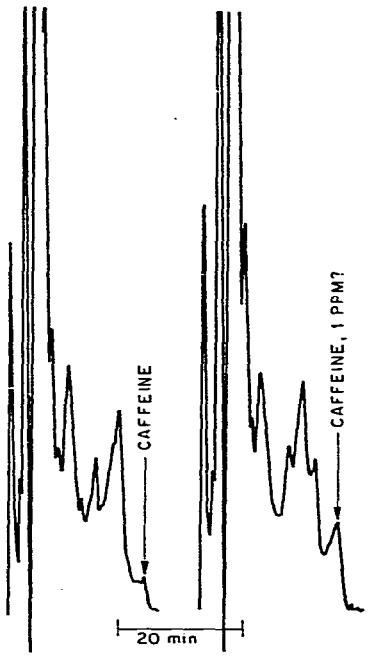


Fig. 10. Chromatograms of deproteinized serum; see text. Conditions as Fig. 9.

CONCLUSIONS

The effects of pH and solvent composition on retention of model compounds can be understood in terms of acid ionization constants, polar and hydrophobic character. The effect of the resin counter-ion is more complex and depends on the physical structure of the resin polymer. Chromatographic separations can be optimized when these effects are known.

ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation Grant No. CHE76-08933. We are indebted to the Hitachi Corp. of Japan for the gift of resins and porous polymers, and to Marilyn Schnibbe and Dr. Kazuko Aramaki for making some of the measurements.

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