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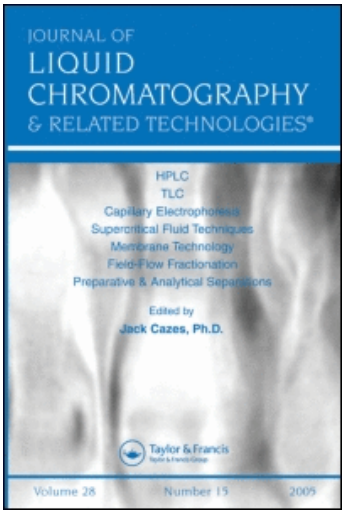

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High Performance Liquid Chromatographic Analyses of Sulphonamides and Dihydrofolate Reductase Inhibitors. I. Separations in Methanol-Modified Solutions

Maria C. Ricci[a]; Reginald F. Cross[a]
[a] School of Chemical Sciences Swinburne University of Technology, Hawthorn Victoria, Australia

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J. LIQ. CHROM. & REL. TECHNOL., 19(3), 365–381 (1996)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSES OF SULPHONAMIDES AND DIHYDROFOLATE REDUCTASE INHIBITORS. I. SEPARATIONS IN METHANOL-MODIFIED SOLUTIONS

Maria C. Ricci,# Reginald F. Cross*

School of Chemical Sciences
Swinburne University of Technology
John Street, Hawthorn
Victoria 3122, Australia

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ABSTRACT

Twenty-two sulphonamides and the three commonly used dihydrofolate reductase inhibitors have been subjected to an investigation of retention behaviour on a silica based reversed phase. Effects of variation in the percentage of methanol and the pH have been determined isocratically and the methanol gradients developed have been modified by variations in the concentration of the phosphate buffer. Significant variations in retention behaviour were observed such that the majority of combinations of drugs could be screened for. No set of conditions studied gave rise to a total separation of all drugs, but around twenty drugs were commonly resolved.

#Current address: Applied Biosystems Division of Perkin-Elmer Corporation, 1270 Ferntree Gully Road, Scoresby, Victoria 3179, Australia.

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INTRODUCTION

Assays for individual sulphonamides (SFA), often in combination with metabolite(s) or a dihydrofolate reductase inhibitor (DHFR) potentiator have been published extensively over the years. The early work in this area was largely concerned with theraputic studies and has previously been reviewed.[1] Since that time, the SFA have become the subject of residue analyses due to the prophylactic use of SFA in veterinary medicine which has led to traces of these drugs in milk, eggs, fish and meat. These studies have also been reviewed,[2,3] but many more recent publications have appeared.[4-22]

On the other hand, there have not been any investigations of instrumental methods for the broad screening for SFA and DHFR published since our capillary zone electrophoretic (CZE) work in 1993.[2] In this study it became apparent that conventional CZE (with the detector at the cathode) could not be successfully used to concurrently analyse for a wide range of SFA - or any other negatively charged compounds - if the span of pK_a values is too great. At low pH, some compounds are unionised and inseparable from each other and the neutral marker, whilst others overcome the electroosmotic flow (EOF) and migrate in the wrong direction. At high pH where all compounds may be ionised the EOF is far too great and the separation space is too small for multi-component mixtures.[23] Control of the EOF, perhaps including its reversal, or the use of micellar electrokinetic chromatography is required.

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In the one recent publication concerning the separation of SFA,[22] micellar liquid chromatography was successfully employed, albiet only for twelve of the drugs. This paper reports the first part of a systematic examination of the HPLC separation and analysis of twenty-two SFA and the three commmonly occurring DHFR.

EXPERIMENTAL

Chemicals and Materials

The twenty-two SFA used in this study are sulphanilic acid (SNAC), sulphaguanidine (SG), sulphabenzamide (SB), sulphisoxazole (SISX), sulphacetamide (SAC), sulphamethizole (SMIZ), sulphachloropyridazine (SCP), sulphaquinoxaline (SQ), sulphamethoxazole (SMOX), sulphadimethoxine (SDIM), sulphadiazine (SDZ), sulphamethoxypyridazine (SMP), sulphameter (SM), sulphamerazine (SMRZ), sulphathiazole (ST),

sulphamethazine (SMAZ), sulphapyridine (SP), sulphanilamide (SAN), sulphamoxole (SAM), sulphisomidine (SISM), phthalyl sulphathiazole (PST) and succinyl sulphathiazole (SST). The three DHFR were diaveridine (DVD), pyrimethamine (PYR) and trimethoprim (TMP). The SFA and DHFR standards were all procured from Sigma (St. Louis, MO, U.S.A.). Their individual structures have previously been given.[2] Standard stock solutions for each drug were prepared by dissolving exactly 50 - 250 mg of the pure standard into 100 mL HPLC grade methanol. Combined standards were evaporated by N_2 purging, diluted with milli-q-water to give a final concentration of 25 ng/μL for each component and filtered with a 0.45 μm filter prior to injection. The resultant MeOH/H_2O ratio of the sample solution was 4:96.

Phosphate buffers were prepared using Na_2HPO_4 and KH_2PO_4 and adjusted to the desired pH with 20% KOH or 20% H_3PO_4. All chemicals were of AR grade and milli-q-water was used to prepare all solutions. Buffers were degassed by vacuum filtration.

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Equipment

The HPLC system was a Varian (Walnut Creek, CA) Vista 5560 Series adapted for packed capillary operation . The pump had a flow rate range of 0.01 to 15 mL/min and was not modified. A tee piece was introduced downstream to split the flow. The bulk of the mobile phase was diverted through the parallel conventional column, leaving several μL/min flow to the packed capillary column. Both columns were housed in the oven of a cut-down Perkin-Elmer (Norwalk, CT) Model LC 65-T thermostatted detector. An air actuated automatic Valco (Houston, TX) 1/16" Model C14M injector with 1 μL sample loop was used at 80 psi nitrogen for all experiments.

The Varian UV-200 UV/Vis detector was modified by the replacement of the standard flow cell of 4.5 μL (4 mm path-length) with a 0.5 μL (0.5 mm path-length) flow cell. All absorbances were measured at 270 nm.

Dispersion in the transfer lines was minimised by mounting the injector at the foot of the oven door and using 10 cm of 0.13 mm (0.005") i.d. stainless steel tubing to the column head. Five cm of 0.005" i.d. tubing connected the column exit to the detector which was mounted directly above the column oven. Chromatographic data was collected, integrated and plotted using a Varian CDS 401/2 Vista Series system.

The Varian-packed, protein C_{18} columns were 30 cm stainless steel, 0.35 mm i.d. and 0.48 mm o.d. The stationary phase was the Separations Group Vydac IDI-TP 5 μm ± 1 μm (75%) silica with surface area 80 m^2/g, pore volume 0.63 cm^3/g and average diameter of 330 Å. The C_{18} bonded phase was TMS capped with a total carbon loading of 6-7%.

Experimental Methods

Flow rates through the packed capillary column were measured by collecting timed volumes of eluate into a 5 mL measuring cylinder via a piece of plastic tubing connected to the outlet of the restrictor column.

Peak identifications were established by adopting a time-saving peak tracking method based upon the minimum number of injections required to elucidate the maximum number of components.[24] In this case a minimum of five samples of varying compositions of the SFA and DHFR was required for full identification.

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Spectroscopic grade KBr dissolved in Milli-q-water was injected to determine the hold-up time (t_0 = 2.83 minutes) and was used as a mobile phase to determine the delay time ($t_d = t_{SOLVENT\ FRONT} - t_0$ = 6.57 minutes). The mobile phase was delivered from the pump at the standard flow rate of 1 mL/min for all the packed capillary column analyses and the detector was set at 270 nm. Under the range of gradient conditions used, equilibration times of 20 minutes were found to be adequate.

Unless otherwise specified, the column oven was set to 30°C and the flow rate at the pump was 1.0 mL/min. This yielded a flow rate of approximately 6 μL/min through the packed capillary column.

RESULTS AND DISCUSSION

In an earlier study,[1] two reversed phases were used. For the primary stationary phase utilised, it was found that significant losses occurred for many of the twenty-five drugs. In the case of the SFA, losses varied from negligible with the early eluting, hydroplilic drugs through to 73% with the most hydrophobic (SQ). Each of the DHFR was totally lost. However, in the same study[1], an alternative stationary phase was also examined. This (10 μm diameter silica) stationary phase gave rise to relative peak areas consistent with the concentrations utilised and the measured molar absorbtivities for all drugs,

indicating that there were not any losses of either class of compound. For this investigation, the identically derivatised, 5 μm diameter silica from the same source was chosen as the stationary phase.

Methanol (MEOH) was chosen as the first organic modifier to test. It appeared to have been less extensively investigated previously and the weaker reversed-phase solvent (compared to acetonitrile) permitted easier experimental fine tuning of the net solvent strength. The initial pH chosen for the mobile phase was based upon previous studies[25,1] in which values of 2.5-3 were found to yield the best available separations (in conjunction with acetonitrile as the organic modifier and acetate or phosphate buffers, respectively). As phosphate buffers yielded the then best separations of sulphonamides obtained,[1] they were again employed in this study.

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Isocratic Analyses

(a) The effect of % methanol

In this section, 0.1 M KH_2PO_4 was adjusted to pH 2.75 and trial chromatograms obtained at 10, 15, 20 and 25% MEOH. From the 25 compounds injected on each occasion, the respective results may be summarised in terms of the respective numbers of discernible peaks (or parts thereof) and the total analysis times: 21 peaks in 270 minutes, 19 peaks in 145 mins., 17 in 70, and, 13 in 40. As is usual with isocratic analyses, there is the trade-off between an acceptable number of separations and an acceptable time of analysis. With a view to later gradient procedures significantly modifying the long analysis times further, additional runs were performed in the intermediate range (15-20% MEOH) where large reductions in analysis time were offset by only modest losses in the numbers of peaks. Figure 1 shows the results of tracking all of the individual drugs at all of the percentages of MEOH examined.

However, it must be kept in mind that the log k' plots are easily over interpreted. For example, in Figure 1, at 15% MEOH with log k' 0.2-0.3, SDZ (open square) and SISM (closed square) appear to be just separated. They are not. Finite peak widths ensure that individual compounds with distinguishable retention times in separate chromatograms may not even appear as 'shoulders' in the mixture. Indeed peak widths for the sulphonamides are a serious impediment to separation. Using a method adapted from the literature,[26] the estimated[27] theoretical plates for the column used in this study was 8550. On the other hand, the realisable plates in the crowded region of the chromatogram (16% MEOH) varies from only 1700 for SAC at log k' = 0.1 to 3000 for SMP at

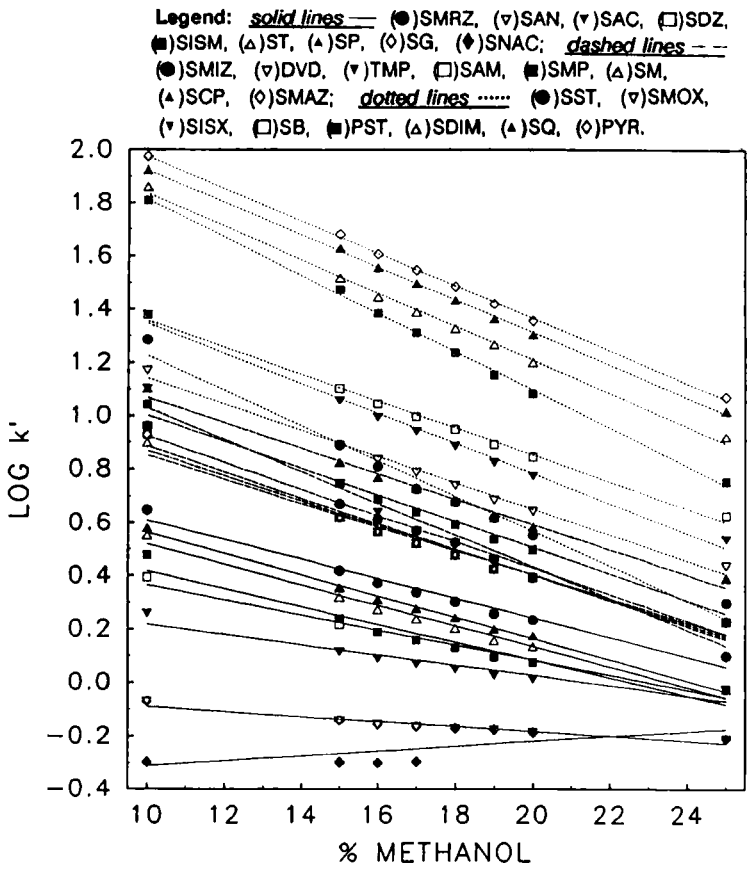

Figure 1. Plot of log k' vs % methanol for the 22 SFA and 3 DHFR compounds using a 0.1M phosphate buffer at pH 2.75.

log k' = 0.7. In Figure 1, at 10% MEOH (with log k' 0.4-0.5) SDZ and SISM appear to be well separated; and in the main, they are. But near the baseline they are not. This is due to the non ideal peak shapes typical of these compounds with highly polar functional groups. Further exaccerbation of the misinterpretation of log k' plots can also arise due to the imperfect lines of best fit drawn by the software.

Nonetheless, the value of the plots is clear in providing an overview. Given the convergence of the log k' plots, one crossover at about 15% MEOH and several others above 17%, 16% MEOH appeared to be the optimum

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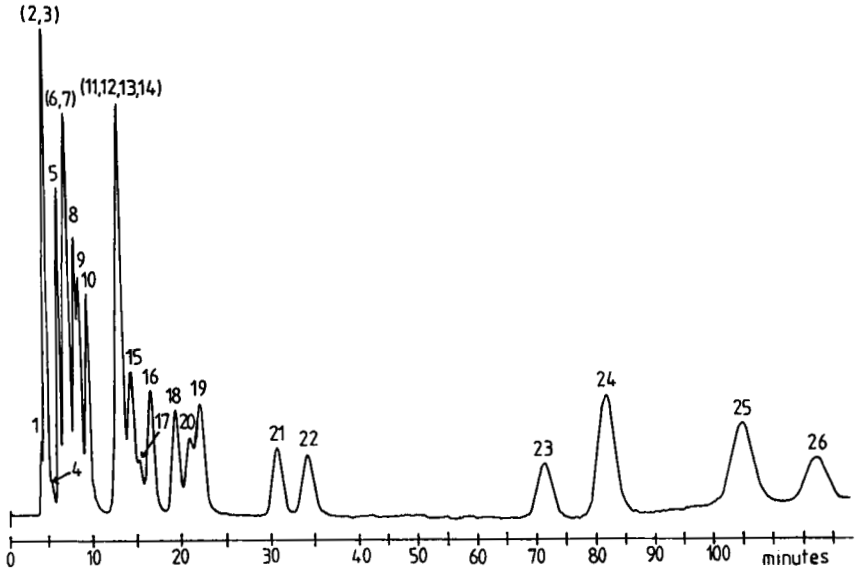

Figure 2. Isocratic 16% methanol/84% 0.1M phosphate buffer (pH 2.75) chromatogram. The compounds are: **(1)**SNAC, **(2)**SG, **(3)**SAN, **(4)**SAM hydrolysis product, **(5)**SAC, **(6)**SDZ, **(7)**SISM, **(8)**ST, **(9)**SP, **(10)**SMRZ, **(11)**SM, **(12)**SAM, **(13)**SMAZ, **(14)**DVD, **(15)**SMIZ, **(16)**SMP, **(17)**TMP, **(18)**SCP, **(19)**SMOX, **(20)**SST, **(21)**SISX, **(22)**SB, **(23)**PST, **(24)**SDIM, **(25)**SQ, **(26)**PYR.

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composition for further studies (especially remembering that the 10% chromatogram took 270 minutes). Figure 2 shows the chromatogram at 16% MEOH.

(b) The effect of pH

As stated above, the initially chosen pH of the mobile phase (2.5-3) was based upon previous studies. However, this is not what would logically be expected on the basis of the known pK_a data. (For structures and pK_a values see reference 2.) Firstly, several of the $pK_{a,1}$ values are in the vicinity of 2.5 so that differential diffusion/migration of the acid-base conjugates would be expected to give rise to band-broadening and loss of resolution. This was found particularly to be the case for SP;[1] $pK_{a,1}$ = 2.6. Secondly, $pK_{a,1}$ values range from 1.5-2.6 and generally[23] refer to the deprotonation of the anilinium group, and, $pK_{a,2}$ values span 4.6-10.4 with the majority between 5.4 and 7.4. With the exception of SA, these $pK_{a,2}$ values refer to the ionisation of the

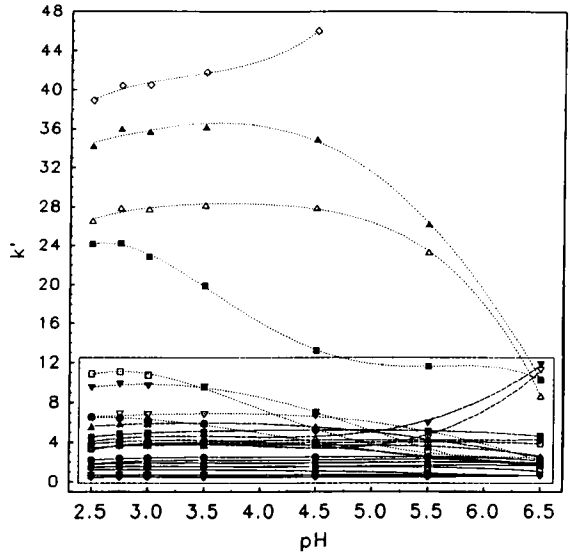

Figure 3(a). Plot of k' vs pH for the 22 SFA and 3 DHFR using isocratic methanol/0.1M phosphate buffer (16:84) conditions. Legend as in Figure 1.

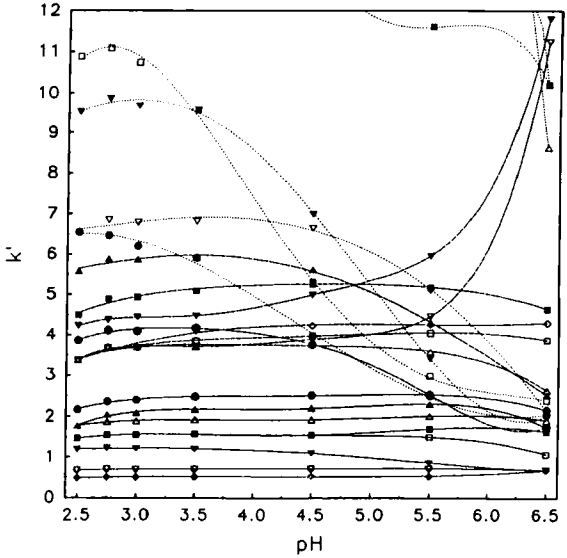

Figure 3(b). Plot of k' vs pH. Enlargement of boxed area in figure 3(a) for the antibiotics in the k' region between 0.0 and 12.0. Legend as in Figure 1.

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sulphonamide nitrogen. Thus, for these moderately polar drugs, maximum retention and separation would be expected in the vicinity of pH 4. Hence, there is good reason to examine the pH dependence of the separation.

All of the runs in this section were carried out in 16% MEOH and 84% aqueous 0.1 M phosphate buffer blended from two reservoirs at pH 2.5 and 6.5. The required proportions were calculated and then verified at the purge outlet. Figure 3(a) shows the plots of k' versus pH for all of the drugs and Figure 3(b) is an enlargement of the crowded, boxed area at the bottom of Figure 3(a). We have chosen to display the data in this way (rather than as the more evenly spaced log k' plot) in order to clearly show the shapes of the variations. (The data is also slightly less crowded.) In all cases where pK_a data is known, the shapes of the retention plots are in accord with expectation.

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Figure 3(b) contains some very encouraging information. Two of the extremely difficult groups of peaks at low pH are separated at higher pH. At k' 1.4 SDZ and SISM separate above pH 5 and the coincident quartet of compounds at pH 2.5 and k' 3.3 (DVD, SAM, SM and SMAZ) move apart as pH increases. Contrary to these gains are the excessive number of crossovers among the retention plots, the number of which increases dramatically with increasing pH. Above pH 4.5, the situation is chaotic. It is unfortunate that several of the moderately retained SFA at pH 2.5 (SB, k'=11; SISX, k'=10; SST, k'=6.5; SMIZ, k'=3.9) have low $pK_{a,2}$ values (4.6, 5.1, 4.16($pK_{a,1}$ but ionising) and 5.4, respectively) so that large losses in retention with increasing pH cause many of the crossovers. DVD and TMP (k' 3.3 and 4.2 at pH 2.5) add to the problem by deprotonating and increasing retention against the trend. Scanning the pH range in Figure 3(b) does reveal some pH windows that appear promising. PH 5.20 is one of these. However, closer inspection of the data is not encouraging.

Firstly, the pH window is very narrow. There are multiple crossovers within $\pm$0.2 pH units. Secondly, again it is important to keep peak widths in mind to avoid over interpretation of the data. Thirdly, some irreproducibility of the experimental data and some inexactness in the graphical lines of best fit must be taken into account. Finally, when it is realised that the difficult to separate pairs at k' 1.5 and 0.7 are still fused, pH 5.2 is not at all attractive. The most logical pH (4) is similarly difficult, although fewer crossovers of retention plots are in that vicinity. The original pH region appeared as good as any. Further runs and close inspection of the chromatograms indicated a marginal preference for pH 2.8.

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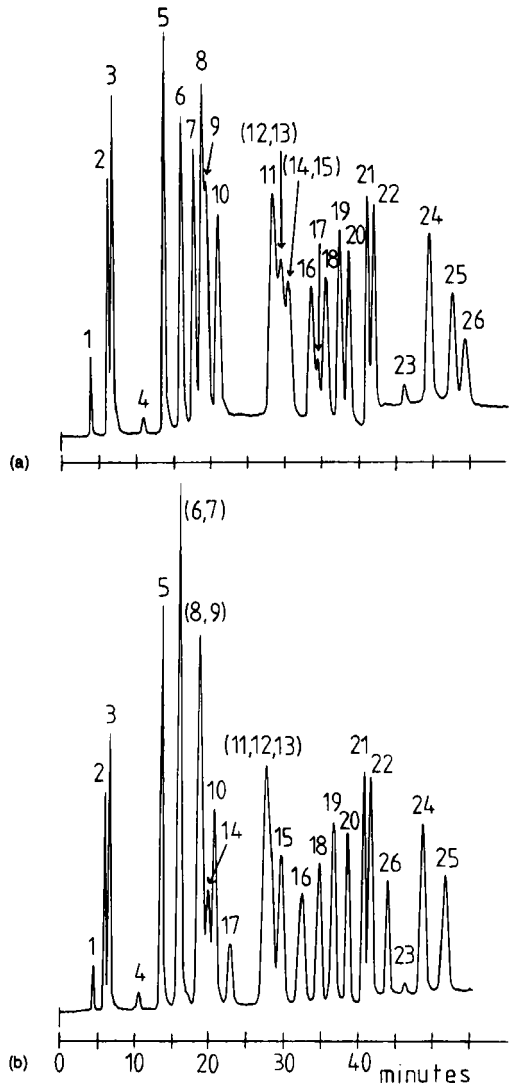

Figure 4. Chromatograms showing the effect of phosphate buffer concentration on the SFA and DHFR separation using gradient #9. The exact pH was 2.72. The numbering of the compounds is as in Figure 2.

(a) 0.1M phosphate buffer. (b) 0.001M phosphate buffer.

Gradient Analyses

(a) 0.1 M phosphate

During the early isocratic separations it became apparent that some of the early eluting peaks were only likely to be separated at greater k' values so that 10% MEOH and less would be required for the initial conditions. By consultation of the log k' versus % MEOH plot, and with the pre-determined delay and column holdup times, a series of solvent gradients were tested and modified. All runs were done at pH 2.8.

Compared to the best compromise isocratic separation (16% MEOH), all of the gradients were highly successfull in the following aspects. (a) Total separation time was reduced from 120 minutes to 50-60 minutes for the solvent programs, plus 15-20 minutes re-equilibration. (b) Although only seen as a leading spike on the combined second (SG) and third (SAN) peaks at 16%, the first peak (SNAC) was always far better than baseline resolved. (c) SG and SAN were always 50-90% separated. (d) Figure 4(a) (gradient details below) also shows the first main group of peaks at t_r 14-21 minutes to be well separated (SAC, SDZ, SISM and SMRZ) with one exception. ST is the peak at 19 minutes and SP is the following shoulder. Under some conditions and on some occasions, the SP peak could be observed as a clear spike on the side of the ST peak. (e) The last eight to nine peaks were always largely resolved.

To obtain the chromatogram in Figure 4(a), a multi-stage gradient in MEOH was employed. Equilibration took place at 0% MEOH and was jumped to 10% at the start of the run. This was followed by linear stages to 12% MEOH at 15 minutes, to 16% at 20 minutes, to 18% at 25 minutes and finally to 30% at 30 minutes. In figure 4(a), from 28-35 minutes, the difficult central section of the chromatogram contained seven compounds in the five peaks: SM, SAM+SMAZ, DVD+SMIZ, SMP and TMP. TMP was generally not resolved from SMP, but did sometimes appear as a shoulder. The first five compounds in this group were clearly the most difficult to separate in the context of the 25 compounds and often appeared as only two peaks.

(b) Effect of phosphate concentration

Figure 5 shows the variation in retention time with (log) phosphate concentration (mM). The 1.0M phosphate run terminated at 28 minutes when the maximum pressure load of 410 atm was exceeded. The 1.0 M solution was too high in salt concentration to be employed with increasing MEOH

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concentrations. The eight compounds that did elute are included. Note that SP is almost baseline resolved from ST. This particular pair had previously been very difficult to separate.

The results in Figure 5 are striking in the dichotomy between the behaviour of the SFA and the DHFR. The former show little in the way of salt effects. In view of the moderate polarity of these compounds and the partial charges carried by the majority, this is not suprising. On the other hand, the effect on the DHFR is relatively dramatic. With $pK_{a,2}$ values for the first protonation of the electrically neutral base from 6.6-7 and $pK_{a,1}$ values for the second protonation around 1.3,[28] the DHFR have greater than one charge and their behaviour must be considered as that of ions in a swamping electrolyte. A plot of t_r versus the square root of the buffer concentration yields a convex curve, the inverse of which would represent the variation of the activity coefficients of the analytes in the increasing concentration of the buffer. This half-parabolic shape is consistent with the expected behaviour, if displaced a little further towards low salt concentrations than expected.

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From the point of view of the separation, the size of the salt effect on the retention of the DHFR is very favourable. In reducing the phosphate concentration from 0.1 M to 0.001 M, (a) the last eluting peak (PYR) is eluted earlier but with baseline resolution maintained, thus reducing the total run time by about 5 minutes. Also, (b) the difficult to resolve TMP is removed to a vacant part of the chromatogram and is baseline resolved. (c) DVD, one of the compounds previously inseparable from the most difficult group of 5 located in the middle of the chromatogram, is moved forward to an earlier group (but causes a deterioration in that region). Figure 4(b) shows the chromatogram in 0.001 M phosphate.

Comparison of Figures 4 (a) and (b) is revealing. At higher salt concentrations the front of the chromatogram is almost resolved, whilst at lower salt concentrations the end of the chromatogram is resolved. Two isoionic elution stages joined by a gradient(s) in salt concentrations across the 20-30 minute period suggests itself. However, the timing and the gradient of the salt programming across this critical central period could be difficult. (Again the significance of peak widths should not be forgotten.)

(c) The final gradient (0.001 M phosphate)

With the improvements observed in the second half of the separation in 0.001 M phosphate, some further investigations of the front end of the gradient

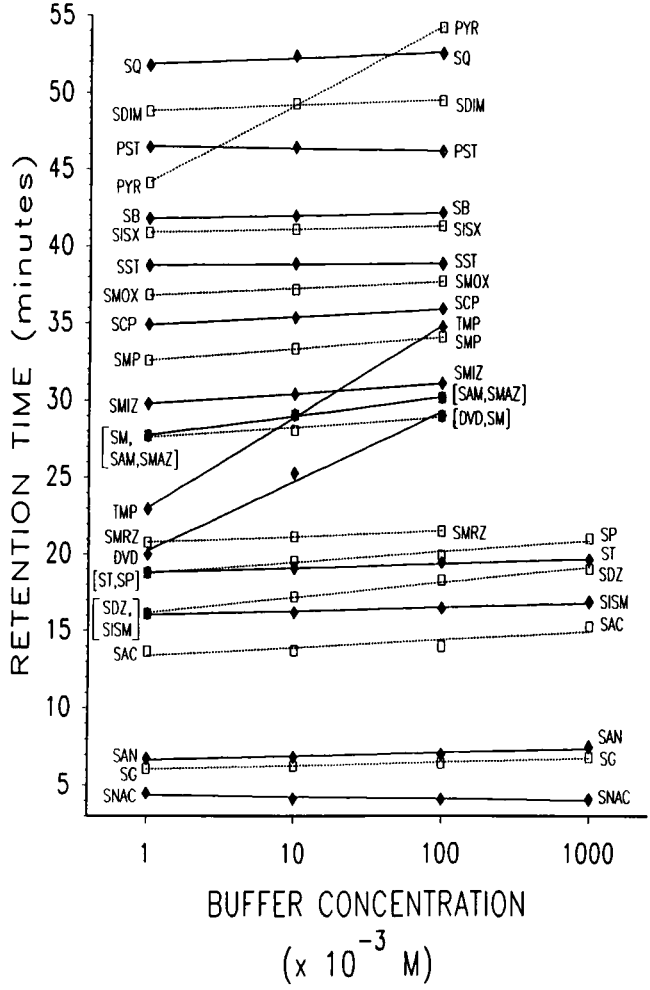

Figure 5. The influence of the variation of the phosphate buffer concentration on the retention time for the 25 antibiotics. Legend as in Figure 1.

were carried out. The best result was obtained by a variation of the gradient described in part (a) of this section. The 10-12% step in MEOH from 0 minutes was extended out to 30 minutes, then to 18% MEOH at 35 minutes and to 30% at 40 minutes. Figure 6 shows the result. Only two coelutions remain, compounds 8 (ST) and 9 (SP) [of which the later appears as a clear shoulder],

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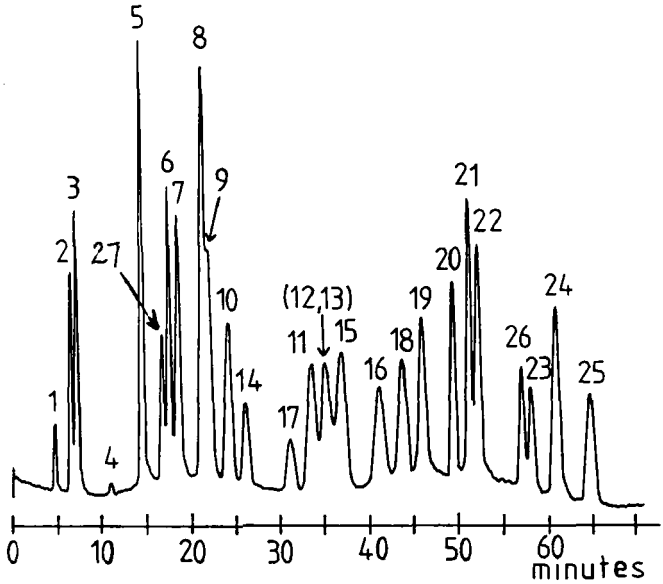

Figure 6. The chromatogram obtained using the final gradient described in the text (0.001M phosphate buffer). The numbering of the compounds is as in Figure 2. **27** is the second SAM hydrolysis product.

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and, compounds 12 (SAM) and 13 (SMAZ). A further advantage lies in the revelation of the second SAM hydrolysis product (27) which had hitherto been unresolved.

CONCLUSIONS

The systematic and detailed examination of the retention behaviour of 22 sulphonamides and 3 dihydrofolate reductase inhibitors indicates that most combinations of the drugs could be successfully screened under an appropriately chosen set of conditions. For example, one of the groups of drugs most difficult-to-separate under the majority of conditions investigated are sulphameter(SM), sulphamoxole(SAM), sulphamethazine(SMAZ), sulphamethizole(SMIZ) and diaveridine(DVD). At pH 6.5 these are baseline resolved. Separation of all 25 drugs simultaneously has not been achieved, but may be possible with simultaneous methanol and salt (or pH) gradients.

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