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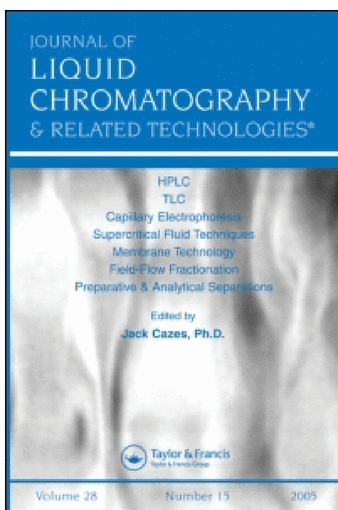

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High Performance Liquid Chromatographic Analyses of Sulphonamides and Dihydrofolate Reductase Inhibitors. II. Separations in Acetonitrile Modified Solutions, Ternary Gradient Studies & Flow Programming

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSES OF SULPHONAMIDES AND DIHYDROFOLATE REDUCTASE INHIBITORS. II. SEPARATIONS IN ACETONITRILE MODIFIED SOLUTIONS, TERNARY GRADIENT STUDIES & FLOW PROGRAMMING

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ABSTRACT

Acetonitrile has been investigated as the organic modifier for the reverse phase separation of twenty-two sulphonamides and three commonly used dihydrofolate reductase inhibitors. Isocratic analyses indicate that 10% acetonitrile is approximately isoeluotropic with 16% methanol. This allows a comparison of selectivities. Relative to methanol, in gradient elution involving acetonitrile only, unfavourable changes in the relative retentions of some pairs of analyses outweighed the advantages of favourable selectivities. Insertion of acetonitrile into methanol gradients did not produce the improved separations expected. However, the imposition of a program of reduced flow upon an

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established methanol gradient demonstrated significantly increased efficiencies in the front half of the chromatogram. This permitted the resolution of the first thirteen compounds, including the difficult-to-separate pair of sulphathiazole and sulphapyridine.

INTRODUCTION

In a previous paper,[1] we presented the results of an investigation into the reverse phase retention behaviour of twenty-two sulphonamides (SFA) and the three commonly used dihydrofolate reductase inhibitors (DHFR) in methanol modified mobile phases. The effects of variation in the percentage of methanol and the pH were determined isocratically. Gradients were developed and then modified by variation of the concentration of the phosphate buffer. Significant variations in retention behaviour were observed such that the majority of combinations of drugs could be screened. However, no set of conditions studied gave rise to a total separation.

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Methanol (MEOH) was chosen as the first organic modifier to examine, as there were no reports of detailed studies of its effects on the broad screening for SFA. Thus, any possible advantages in selectivity were unknown. Furthermore, the weaker reverse phase solvent (compared to acetonitrile (ACN)) permitted easier experimental fine tuning of the net solvent strength. However, whilst the MEOH work revealed sets of conditions conducive to most separations, the ultimate aim of the separation of all analytes was not achieved. Thus, we returned to ACN. ACN has been investigated as an organic modifier in the separation of the SFA before,[2,3] albeit not in conjunction with the particular stationary phase utilised in this study.

Given appropriate selectivity differences, there were two possible useful outcomes. The first was an outright separation of all of the drugs in ACN. The second possibilty was to incorporate ACN into a MEOH gradient if sufficient difference was evident between the solvents in the critical part(s) of the separation. In all attempts to separate a large number of the SFA, the major stumbling block has been those SFA eluted at moderate solvent strengths in the middle of the chromatogram. As this includes previous ACN studies (on other stationary phases), it was prudent to consider an alternate strategy.

Adjustment of the flow rate is generally regarded as an ancilliary tool. In its simplest application the last one or two strongly retained analytes may be more rapidly eluted.[4] This is particularly useful in isocratic analyses since

pressure (and thus flow) re-equilibration is rapid. None of the column characteristics, nor the nature of the variation of the plot of the height equivalent to a theoretical plate (H) versus flow rate,[5] nor the initial position on the curve are important.

Loss of efficiency is irrelevant if the analysis time is significantly reduced. At the front end of the chromatogram, additional separation time is provided by reducing the flow rate. Whilst the same increase in separation space may be achieved by an appropriate reduction in the solvent strength, we have experienced the combination of analyses and stationary phase for which the former provided a separation but the latter did not.[6] In this case the initial position on the H/flow rate curve does matter.

Excessive peak broadening due to the ascent of the steep section of the H versus flow rate plot at very low flow rates could be counter productive. Fortunately, the commonly adopted flow rates are often above the optimum so that we are protected from that eventuality. Flow rate programming has also been used for some time as the solution to the general elution problem in the analysis of compounds which are only conveniently detected by refractive index.[7] The general coupling of solvent and flow programming has also been systematically examined.[8]

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EXPERIMENTAL

With the exception of the HPLC grade ACN, all chemicals, equipment and experimental methods were as previously described.[1]

The pH range of 2.5-3, found to be optimal by Roos,[2] and confirmed in Part I of this study, was used without further investigation. Similarly, the phosphate buffer found to yield the best separations in Part I of this study (0.001 M), was also utilised without further study.

Although not reported in Part 1, substitution of phosphate by acetate (in methanolic solutions) yielded poorer separations. The 0.001M KH_2PO_4 buffer solutions were adjusted to the pH range of 2.75-2.77 and the column oven was set to 30°C for all runs unless otherwise indicated.

The full names of the compounds are given in the first part of this study[1] and the structures of all of the drugs have also been listed previously.[9]

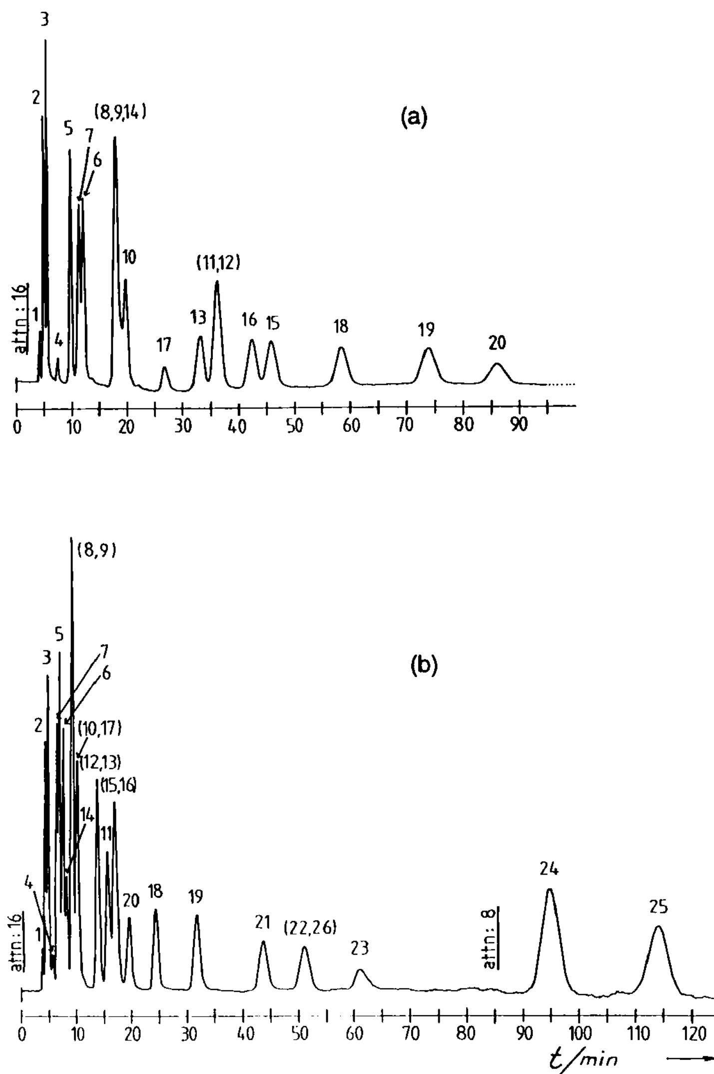

Figure 1. Isocratic acetonitrile (ACN)/0.001 M phosphate buffer (pH 2.75) chromatograms. (a) 5% ACN (thefirst 90 minutes). (b) 10% ACN.
The compounds are: (1)SNAC, (2)SG, (3)SAN, (4)SAM hydrolysis product, (5)SAC, (6)SDZ, (7)SISM, (8)ST, (9)SP, (10)SMRZ, (11)SM, (12)SAM, (13)SMAZ, (14)DVD, (15)SMIZ, (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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Table 1

Comparison of the Isocratic Elution Characteristics for the 22 SFA and 3 DHFR in 16% Methanol and 10% Acetonitrile

Time (Minutes)	16% Methanol (0.1M Phosphate Buffer, pH 2.8)	10% Acetonitrile (0.001M Phosphate Buffer, pH 2.8)	
4.5	SNAC(1)	SNAC(1)	
5	SG(2)&SAN(3)&SAM(HP)(4)	SG(2)	
		SAN(3)	
		SAM(HP)(4)	
6.5		SISM(7)	
7	SAC(5)	SAC(5)	
8	SDZ(6)&SISM(7)	SDZ(6)	
9	ST(8)		[DVD(14)]
	SP(9)		
10	SMRZ(10)	ST(8)&SP(9)	
10.5		SMRZ(10)&....	[TMP(17)]
14	[DVD(14)&..SM(11)SAM(12)&SMAZ(13)		
14.5	SMIZ(15)	SAM(12)&SMAZ(13)	
15	[TMP(17)]		
15.5		SM(11)	
17	SMP(16)		
17.5		SMIZ(15)&SMP(16)	
20	SCP(18)	SST(20)	
21	SST(20)		
22	SMOX(19)		
25		SCP(18)	
31	SISX(21)	SMOX(19)	
35	SB(22)		
44		SISX(21)	
51		SB(22)&...........	[PYR(26)]
71, 61	PST(23)	PST(23)	
82, 95	SDIM(24)	SDIM(24)	
105, 115	SQ(25)	SQ(25)	
123	[PYR(26)]		

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RESULTS AND DISCUSSION

Analyses in Acetonitrile Modified Mobile Phases

(a) Isocratic

Three trial chromatograms were run. The ACN content of the mobile phase, the number of discernable peaks and the total analysis times were 5%: 22 peaks in 430 minutes, 10%: 21 peaks in 120 minutes and 15%: 16 peaks in 40 minutes, respectively. Figure 1(a) shows the chromatogram for the first 90 minutes of the 5% ACN run and Figure 1(b) shows the 10% run. The elution

characteristics for the 10% ACN isocratic run are compared with those for 16% MEOH[1] in Table 1. Column 1 is an approximate time scale with the later double times being those in 16% MEOH and 10% ACN, respectively. Columns 2 and 3 list the peaks in order of elution in 16% MEOH and 10% ACN, respectively. Individual compounds are listed where they are clearly discernable from neighbouring peaks. Otherwise, coeluting compounds are listed together, joined by an ampersand (&). In each list (columns 2 and 3) the DHFR have been put to the side since the observed differences in their elution characteristics have been demonstrated (near the conclusion of the first part of this study[1]) to be due to the decreased phosphate concentrations. Whilst there were changes in the relative retentions of several pairs of SFA when the phosphate buffer concentration was decreased from 0.1M to 0.001M, there are no changes in elution order amongst them.

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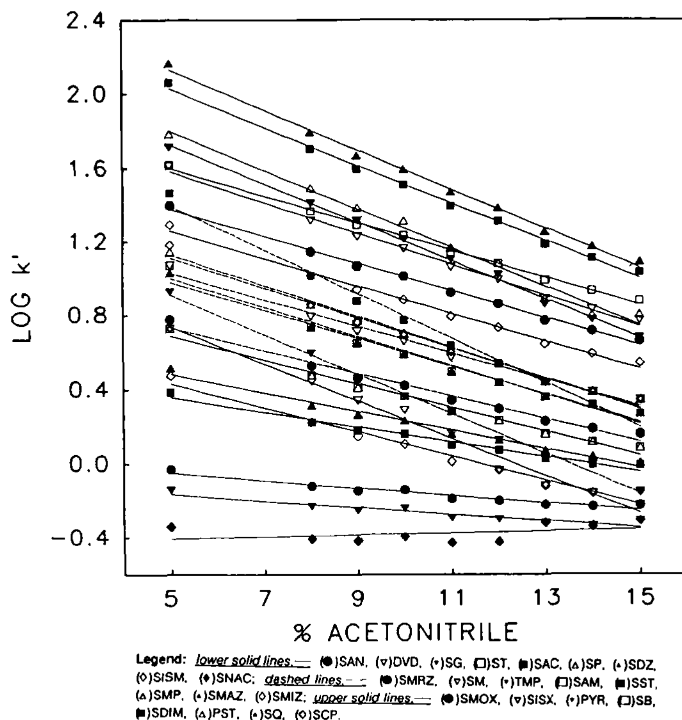
A comparison of the retention data for the 16% MEOH and 10% ACN ioscratic chromatograms indicates these mobile phases to be approximately isoeluotropic. For the 16% MeOH run, a total of 20 peaks were separated in 125 minutes with a k' range of 0.5 - 43. Similarly, the 10% ACN run yielded 21 peaks in 114 minutes with a k' range of 0.4 - 41. Inspection of Table I reveals that the order of elution of the SFA is largely the same in both of the organic modifiers. Of the 22 SFA and the SAM hydrolysis product observable in these runs, 20 were clearly eluted in the same order. In ACN, SISM and SM are eluted earlier and later in that order, respectively, (and are underlined and in bold typeface in the table). SCP and SST are inverted in the elution orders (and are each singly highlighted in the table). Also, closer inspection of Table I reveals several changes in the relative retentions in ACN.

It should be noted from Table 1, however, that with approximately the same number of discernable peaks in each solvent system, the advantages of elution in 10% ACN (compared to 16% MEOH) were largely negated in other parts of the separation. Overall, the most positive change occurred around the 14 minute region of the chromatogram, from which DVD is removed, by the decreased salt concentration, and SM is removed by the differing selectivity of ACN. Since this had been the most difficult region of the separation, it was decided to investigate straight ACN separations.

Isocratic analyses were performed at 8-14% ACN, inclusive, and combined with the earlier data at 5, 10 and 15%. Figure 2 is the plot of log k' versus percentage ACN. At the high percentage ACN end, the plot is compressed and multiple coelutions occur. Working back from 15% ACN towards the less congested regions of the plot, there is another unfortunate feature of the data that becomes apparent. This is the large and non-systematic



Legend: *lower solid lines.*— (●)SAN, (▽)DVD, (▾)SG, (□)ST, (■)SAC, (△)SP, (▴)SDZ, (◇)SISM, (◆)SNAC; *dashed lines.*- - (●)SMRZ, (▽)SM, (▾)TMP, (□)SAM, (■)SST, (△)SMP, (▴)SMAZ, (◇)SMIZ; *upper solid lines.*— (●)SMOX, (▽)SISX, (▾)PYR, (□)SB, (■)SDIM, (△)PST, (▴)SQ, (◇)SCP.

Figure 2. Plot of log k' vs % acetonitrile for the 22 SFA and 3 DHFR compounds using a 0.001M phosphate buffer at pH 2.75.

variation in d(log k')/d(% ACN) for many of the compounds. In the lower section of Figure 2, the gradient of the SISM plot is significantly greater than would be expected in that part of the figure. Slightly higher up in the figure, in the middle region, DVD, TMP and SST have gradients that are even greater, relative to the average at that k'. And, in the band of plots from log k' ≈ 1.6 at 5% ACN down to log k' ≈ 1 at 15% ACN, the gradients for the four compounds (SISX, SB, PYR and PST) vary around the values that would be anticipated in that part of the figure. This is a feature of the ACN analyses that is quite different from the MEOH data where far fewer compounds varied greatly from the overall trend in gradients. The effect of these variations is to cause multiple crossovers in the plots and a large number of coelutions between continually varying pairs of compounds.

From the severely congested end of Figure 1, around 15% and down as far as (and including) 8%, the result is that isocratic analysis looks very

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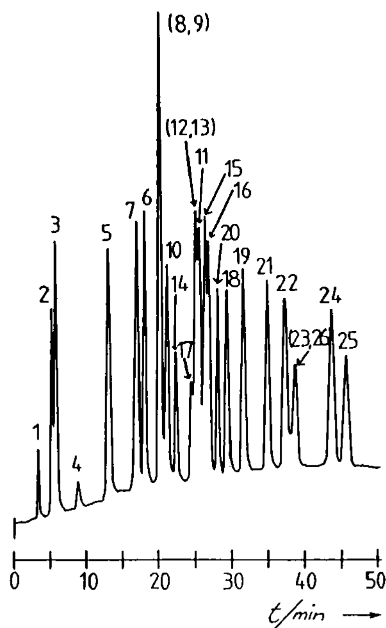

Figure 3. The best gradient chromatogram obtained with ACN/0.001M phosphate buffer (pH 2.75). The percentage of ACN varied with time as: 0 minutes, 0%; 5 min, 5%; 20 min, 15%; 35 min, 18%; 45 min, 30%; 55 min, 60%. The compounds are numbered as in Figure 1.

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unattractive. At 7% ACN there is a narrow window of modifier concentration free of crossovers and, therefore, potentially useful. However, there are two persistent pairs of coeluting compounds (SAM(12) & SMAZ(13) and SMIZ(15) & SMP(16)) and the presumption of no other coelutions depends upon the accuracy of the software-drawn lines of best fit that appear not to be in good agreement with much of the 5% retention data. Furthermore, interpolation of the SQ plot to 7% ACN yields a total analysis time of 238 minutes, so no further investigations of isocratic ACN separations were attempted.

(b) Gradients

One of the early gradients to show promise was a simple linear variation from 0-15% ACN over 29 minutes, followed by isocratic elution. Compared to

Table 2

Gradient Program used for the Chromatogram Shown in Figure 4(b)

Time (Minutes)	%0.001M Phosphate	%MEOH	%ACN
0	100	0	0
0.1	95	5	0
7	92	8	0
7.01	94	0	6
21	94	0	6
26	84	16	0
31	82	18	0
36	70	30	0

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the 10% isocratic separation, there was not any increase in the number of compounds resolved - and amongst the unresolved compounds - two of the most refractory coelutions remained (ST(8) & SP(9) and SAM(12) & SMAZ(13)). However, the total elution time for the 21 peaks was reduced from 120 minutes to 67.

After a large number of variations, the best gradient involved steps from 0-5-15-18-30-60 % ACN at 0, 5, 20, 35, 45 and 55 minutes, respectively. Figure 3 is the chromatogram obtained. With a further reduction of 20 minutes, two additional peaks were obtained. However, the cost of the faster separation was reduced resolution between some pairs. As this separation was still clearly inferior to the best acieved in MEOH, ACN was abandoned as a single modifier. The very high mobility with respect to % ACN of several of the analyses relative to the majority makes the estimation of the net effect of several gradient steps extremely difficult.

(c) Insertion in a methanol gradient

Towards the section (a), it was noted that an inconsistency existed between the extrapolated lines of best fit to the 15-8% ACN isocratic runs and the observed data at 5% ACN for several of the analytes, thus suggesting a possible systematic error in the latter. Aiming specifically at the difficult middle group of compounds (11-16), a 6% isocratic run was performed. Figure 4(a) is the chromatogram for the first 50 minutes and the middle group are all

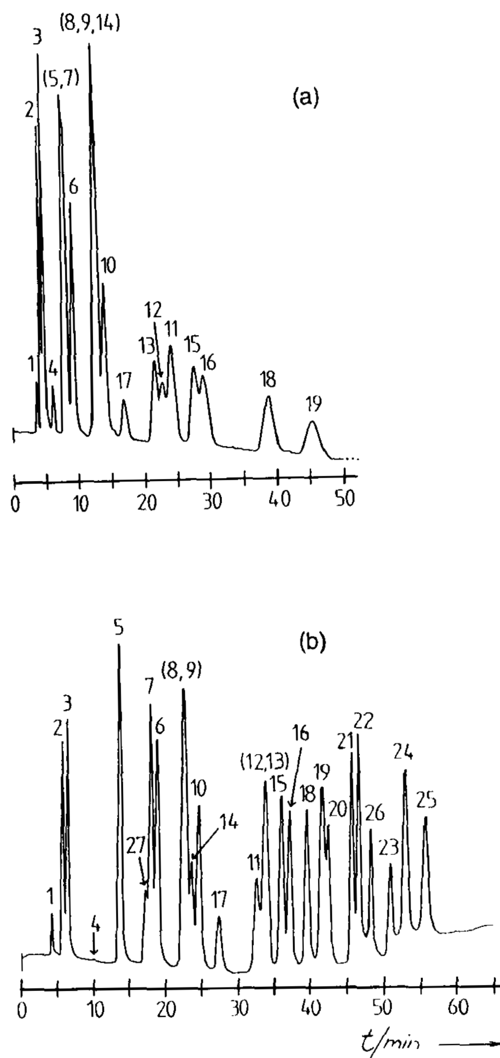

Figure 4. Chromatograms showing (a) isocratic 6% ACN elution and (b) the effect of insertion of 6% ACN in a MEOH gradient. The gradient is given in Table 2. The compounds are numbered as in Fiqure 1. 27 is the second SAM hydrolysis product.

partially resolved. The objective was to maintain the basic features of the best MEOH gradient but to replace MEOH with 6% ACN to elute compounds

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11-16. Eventually, the gradient in Table 2 was settled upon. Figure 4(b) is the chromatogram. With a delay time of 6.57 minutes, the introduction of the 6% ACN at 7 minutes brings the ACN to the column head at 13½ minutes. The effect of this solvent switch is clearly evident around 19 minutes, where the reversal of compounds 6 and 7 is observed, in agreement with Table 1. This implies that the methanol gradient in the first 7 minutes was ineffectual in moving compounds 6 and 7 along the column. This might be expected to apply to the subsequent elutions as well. However, this is far from the case.

Comparison of Figures 4(a) and (b) indicates that MEOH causes the partial resolution of compound 14 from 8 & 9 and the improved separation of 10 from this group. Furthermore, compound 11 elutes before 12 - as is the case in methanolic solutions - rather than after compound 13 (as in ACN; see Table 1). Unfortunately, the difficult-to-separate-pair of compounds, 12 and 13, also reflect the influence of the MEOH and are again coeluted.

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Increasing the Efficiency of the Separation

From the resolution equation,

$$R = 1/4\sqrt{N} \bullet \frac{\alpha - 1}{\alpha} \bullet \frac{k'}{k'+1}$$

it is clear that resolution may be improved by increasing any of N, α or k'. To this point in the investigation, we have largely been concerned with α and k'. The studies of the effect of percentage organic modifier are mainly an examination of variation in k' (with some changes in α), whereas the study of the effect of pH[1] is primarily looking at variations in selectivity (α) (with k' effects also). The change of organic modifier (MEOH to ACN) over the same acceptable range of k' values is purely an investigation of α.

Another option is to increase N. The most obvious way to do this is to increase the length of the column. In a previous study,[3] on a different stationary phase, columns of twice the initial length were prepared and the theoretical increase of 1.4 in the resolution was observed for the SFA. This increase in resolution is highly significant and would clearly be sufficient to resolve any refractory pair of compounds, once they were made discernable in the chromatogram as a two-compound peak, under any set of conditions.

However, the cost is in the doubled analysis time, which is generally unacceptable. A possible compromise involves using an increased flow rate through the longer column.

For a typical monomeric C_{18} bonded phase on microporous 5 μm silica, a doubling of the flow rate, from the vicinity of the optimum value, would only cause approximately a 15% decrease in efficiency.[10] Thus the net effect of doubling both the column length and the flow rate would be to totally offset the increased analysis time and retain a 25% increase in resolution. However, with the 60 cm packed capillary columns prepared for the earlier study,[3] excessive back pressures on the pump prevented this option. Similarly, the 60 cm packed capillary columns prepared for this study again caused excessive back pressures on the pump and could not be utilised in the desired fashion, especially with the more viscous methanolic solutions. As we wished to investigate increasing N whilst retaining the same stationary phase packing, adjustment of the flow rate remained the only available option.

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Flow Programming

For the packed capillary columns used in this study (0.35 mm id), it is difficult to predict the effect of change in the flow rate from the literature. Much of the published data is based upon pioneering research which ranged over a large number of variables. The data of Dong and Grant[10] clearly demonstrated the progressive effect of reduction of particle size (10-5-3 μm) in conventional HPLC, and the relative shapes of the van Deemter plots provide the long accepted basis for fast LC. Similar data has been presented by Verzele and Dewaele,[11] for C_{18} phases bonded to 8, 5, 3 and 2 μm silica. However, at a fixed particle size (5 μm in this case) the extrapolation of height equivalent to a theoretical plate (H) versus flow rate (v) from conventional to capillary packed columns is problematic. Firstly, there is the difference between the reference data. In each case H_{min} is approximately 12 μm and approaches the theoretical limit of 2dp for efficiency, thus indicating that both columns are well packed[12] (dp = the diameter of the packing material.). However, the optimum values of the flow rate (v_{opt}) are significantly different; 1.2[10] and 0.7[11] mL min^{-1}, indicating, in this case, differences in mass transfer characteristics and the quality of the base silica.[12]

Secondly, there is the problem of extrapolation. The expected gains in efficiency due to more homogeneous packing and more uniform heat dissipation in packed capillaries[13] may also shift v_{opt}. However, Kucera's

excellent data[14] for 10 μm silica shows identical v_{opt} in 4.6 and 1.0 mm i.d. columns. Kucera's data indicates that, all other things being equal, v_{opt} should be the same for our packed capillary column as for the conventional columns containing 5 μm particles: Dong and Grant[10] 1.2 mL min^{-1}, or Verzele and Dewaele[11] 0.7 mL min^{-1}, in each case for 4.6 mm i.d. columns. Scaling this flow rate down to the equivalent for our 0.35 mm i.d. column predicts $v_{opt} \approx 7$[10] or 4[11] μL min^{-1}. As the measured flow rate was around 6 μL min^{-1}, with $v_{opt} \approx$ 7 μL min^{-1}, any significant change in the flow rate would be expected to give rise to a decrease in N and loss of resolution. On the other hand, with $v_{opt} \approx 4$ μL min^{-1} the flow rate could be reduced by >50% and efficiency gained. It is clear that, without detailed knowledge of the silica substrate, packing procedures, etc., prediction of v_{opt} is of little use.

Interestingly, for a 10 μm ODS stationary phase, Hirata and Jinno[15] found the H/v plots for a 0.12 mm i.d. column to be higher-lying than the 0.15 mm ≈ 0.25 mm which were above the 0.20 mm i.d. column which achieved $H_{min} \approx$ 2dp). Importantly, v_{opt} appeared to be independent of column diameter at approximately 0.28 mm s^{-1}. Allowing for the difference in particle size between that study (10 μm) and this one (5 μm), via the observed shift in the optimum linear velocity (v_{opt}) for conventional columns,[10] the expected v_{opt} for our packed capillary column would be <5 mm s^{-1}. With t_o = 2.83 minutes and a 30 cm long column in this study, the standard linear velocity used was 18 mm s^{-1}, thus indicating that the flow rate could be reduced by >3 before v_{opt} was reached. Subsequent experimentation was found to be consistent with this. The conclusions to be drawn from these comparisons are that the mass transfer characteristics of our stationary phase are comparable with those for the better published data[11,15] and superior to others.[10] The quality of the packing procedure cannot be evaluated since H_{min} was not determined.

Rather than determine the value of v_{opt}, the flow rate was decreased to see if the separation was improved, and, when this was seen to be so, a process of trial and error was invoked. The flow rate was initially reduced to maximise separation of the most difficult pair (ST(8) & SP(9)) and then adjusted to try to optimise the rest of the separation. Figure 5 shows the final result for the combined flow (f) and solvent(s) program (p) shown in Table 3. The effect on the front end of the separation is as desired with all compounds separated; including ST, SP and the second SAM hydrolysis product(27). We were unable to extend that quality of separation to the rest of the compounds in a reasonable total analysis time.

It is clear that reductions in the flow rate down to 40% of the standard 6

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Table 3

Gradient Program used for the Chromatogram Shown in Figure 6

Time (Minutes)	% 0.001M Phosphate	%MEOH	Pump Flow Rate mL/min
0	100	0	0.4
0.01	95	5	0.4
17	91	9	0.6
30	88	12	0.6
35	82	18	0.4
45	40	60	0.8
50	40	60	0.6
55	40	60	1.0

$\mu L\ min^{-1}$ still yielded increased efficiency. Figure 6 is a plot of N(fsp) versus N(sp) for each of the analytes (mostly) resolved. The solid line corresponds to N(fsp) = N(sp) and no gain in efficiency due to fp. The dashed line is an approximate line of best fit. N(fsp) values were estimated from the chromatogram in Figure 5 for which the combined flow and solvent program is given in Table 3. N(sp) was calculated from the (related) chromatogram obtained without flow programming. (See ref. 1, Figure 6 for the chromatogram.) The solvent(s) only gradient program (p) was 0-10% MEOH at 0.01 minutes, to 12% at 30 minutes, then to 18% MEOH at 35 minutes and to 30% at 40 minutes. Comparison of this program with Table 3 shows that the fsp used a weaker or equal solvent strength up to 35 minutes. After that, it was necessary to utilise a greater solvent strength to achieve a similar total analysis time. However, from 35 minutes plus the delay time of 6.57 minutes and 2.83 minutes of hold-up time (≈ 44½ minutes) or soon afterwards, the effect of the increased solvent strength in the fsp will override the effect of the reduced flow rate and the elution pattern will alter.

In Figure 5, it seems clear that this transition took place before the elution of compound 14. It is only the compounds eluted earlier for which the effect of fp may be isolated. Thus, compounds 1, 2, 3, 5, 27, 6, 7 and 10 are eluted with increased plate counts due to the reduced flow rate, only. Of these, it is also significant that compounds 1, 2, 3 and 10 are among those eluted with the

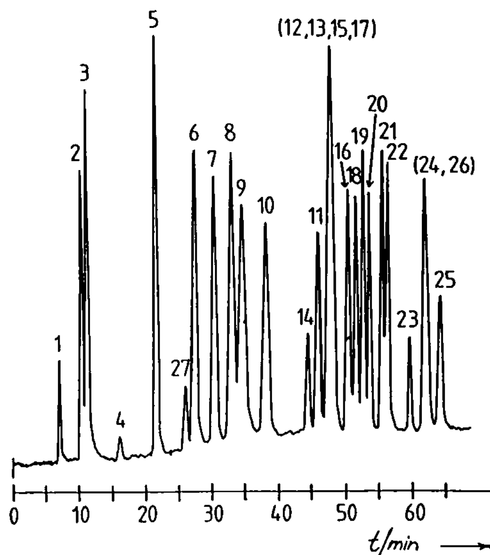

Figure 5. The chromatogram obtained using combined flow and solvent programming as given in Table 3. The numbering of the compounds is as in Figure 1. 27 is the second SAM hydrolysis product.

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greatest increase in efficiency relative to the average (dashed line). These are the compounds in the retention time regions that experienced the lowest average flow rates. It is also especially significant for compounds 1, 2, 3, 5, 27, 6 and 7 because of the weaker solvent strength at the start of the fsp. This would lead to relative peak broadening and tend to counter the gain in efficiency due to moving down the N/v curve towards v_{opt}. The magnitude of the increased efficiency due to fp is easy to under-estimate on the log-log scale necessarily used to equally show all of the data. For example, for representative compounds 1, 3 and 5, N(fsp) is 1340, 2070 and 6850 compared with N(sp) of 510, 1150 and 4400, respectively.

CONCLUSIONS

Examination of ACN as an alternative organic modifier to MEOH in the separation of 22 sulphonamides and 3 dihydrofolate reductase inhibitors has revealed some differences in selectivity, particularly in the most difficult part of the separation. In general, however, this was more than countered by losses of

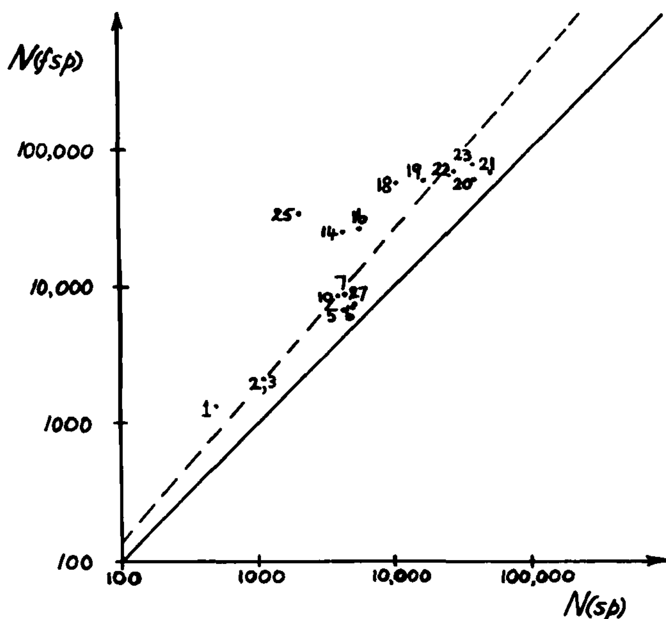

Figure 6. The influence of flow programming on the number of theoretical plates(N) for the compounds (mostly) resolved in the two chromatograms compared. N(fsp) values were estimated from the chromatogram in Figure 6 for which the combined flow (f) and solvent (s) gradient program (p) is given in Table 3. N(sp) was calculated from the chromatogram obtained without flow programming. (See ref. 1. Figure 6 is the chromatogram and the text gives the solvent(s) program(p).) The compounds are numbered as in Figure 1.

resolution due to changed relative retentions in other parts. Attempts to incorporate the benefits of ACN into MEOH gradients were unsuccessful and a complex dependence upon the effects of the two organic modifiers was indicated. On the other hand, concurrent flow and solvent programming clearly demonstrated the positive effect of reduced flow rates and plate heights in the front half of the separation. One of the pairs of drugs most difficult to separate under the majority of conditions investigated are sulphathiazole (ST,8) and sulphapyridine (SP,9). This pair was 2 of the first 13 compounds to be eluted, all of which were resolved. Separation of all 25 drugs simultaneously

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may be possible with flow programming, but only with excessively long analysis times.

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