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Note

Narrow-bore high-performance liquid chromatography separations of 22 sulfonamides

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Sulfonamides (sulfa drugs) have been analysed successfully by thin-layer chromatography (TLC) for several decades[1], and, with the more recent development of high-speed automated TLC scanners, quantitation has been greatly aided.

However, sulfonamides have a strong absorption band (molar absorptivity *ca.* 10^4 l mol^{-1} cm^{-1}) with a maximum between 250 and 280 nm, frequently at about 270 nm. Consequently, it is not surprising that high-performance liquid chromatography (HPLC) with ultraviolet absorption detection has been investigated extensively for the analysis of these drugs. Several studies have been concerned with monitoring levels of a sulfonamide with metabolite(s) and/or a dihydrofolate reductase inhibitor (DHFR) in biological samples[2–13]. Pharmaceutical assays have also been published[14,15]. In addition, there have been some selectivity studies[14–22]. The stationary phases used include ion-exchange[17], amino[2], cyano[14,15], silica[3,5,8,9,19], octadecylsilane[4,6–8,10–13,15,17,18,20] and porous copolymers[21,22]. A complete and detailed overview can be obtained from three recent reviews[23–25].

Good separations of a wide range of sulfonamides have not been obtained.

To achieve greater resolution in HPLC it is necessary to attack the limiting feature of the technique, namely, the limited number of plates available. In this study a 3-μm reversed phase was the most efficient phase available. To maximise the sensitivity and facilitate greater column lengths, capillary columns were chosen.

As sulfonamides are frequently used in conjunction with a DHFR, those available were included in the study. DHRFs have a weak absorbance band (20–30% of the sulfonamides) in the vicinity of 270 nm. A list of sulfonamides and DHFRs was compiled from the Centre for Veterinary Medicine, U.S. Food and Drug Administration priority list of 40 drugs for analytical methods development[26] (including 9 sulfonamides), the Index of Veterinary Specialities[27] and the various investigations mentioned above.

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EXPERIMENTAL

Instrumentation

A Varian (Palo Alto, CA, U.S.A.) Model 5560 high-performance liquid chromatograph and a Varian Model 9050 ultraviolet detector modified with a Varian 0.5-μl (0.5 mm pathlength) flow cell were used along with a Valco submicroliter injector (0.5 μl insert) and a Varian Model 402 Vista data system. To achieve suitably low flow-rates with the capillary column, a split-column configuration was adopted. An air oven was used (Spark Holland Model SpH99) and the micro flow meter was by Phase Sep.

Chromatography

The 30 and 60 cm long capillary analytical columns were 0.014 in. (0.35 mm) I.D. $\times$ 0.019 in. O.D. (syringe needle) stainless steel packed with MicroPak SP C_{18}-3 Phase Sep (Spherisorb) 200 m^2 g^{-1} 3-μm silica C_{18} bonded and capped with trimethylsilane (TMS) to yield 12–13% carbon. Mobile phase conditions were standardized to a flow-rate of 5.5 μl min^{-1} and reservoirs A, water; B, acetonitrile; C, acetic acid–acetonitrile–water (1:12.5:86.5) (pH 2.75). Isocratic analyses used mobile phase C.

Sigma (St. Louis, MO, U.S.A.) supplied all of the sulfonamides and DHFRs.

The 30 cm $\times$ 1 mm microbore column used contained the Micropack MCH-10 C_{18} packing: The Separations Group Vydac (IDI TP) 80 m^2 g^{-1} 10-μm silica C_{18} bonded and capped with TMS to yield 6–7% carbon. A flow-rate of 0.2 ml min^{-1} was adopted for the microbore column and the isocratic mobile phase was from reservoir C as detailed above.

A standard mixture was prepared by weighing 1-mg amounts of each of the 22 sulfonamides and 3 DHFRs, combining these, dissolving in acetonitrile–water (5:95) and filtering. A 25-ng amount of each compound was injected unless otherwise stated. All absorbances were measured at 270 nm.

RESULTS AND DISCUSSION

Preliminary runs were carried out on the 1-mm microbore column. Fig. 1 is the chromatogram of 1 ng of each of the sulfonamides (with the exception of sulfanilic acid) and the DHFRs. A comparison with the best separation previously obtained is as

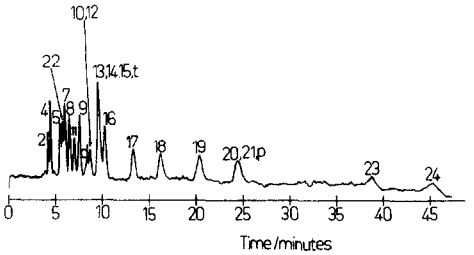

Fig. 1. Isocratic analysis of the standard mixture of 1 ng of each of 21 sulfonamides and 3 DHFRs on the 1 mm microbore column. Identifications for the sulfonamides as in Fig. 2. For the DHFRs: d = diaveridine; t = trimethoprim; p = pyrimethamine.

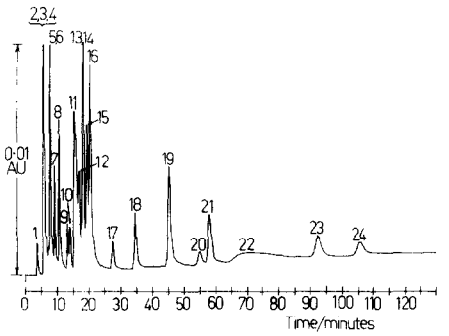

Fig. 2. Isocratic analysis of the standard mixture of 25 ng of each of the 22 sulfonamides and 3 DHFRs on the 30-cm capillary column. The order of elution was: 1 = sulfanilic acid; 2 = sulfaguanidine; 3 = sulfamoxole hydrolysis product 1; 4 = sulfanilamide; 5 = sulfacetamide; 6 = sulfamoxole hydrolysis product 2; 7 = sulfadiazine; 8 = sulfathiazole; 9 = sulfamerazine; 10 = succinyl sulfathiazole; 11 = sulfapyridine; 12 = sulfamoxole; 13 = sulfameter; 14 = sulfamethizole; 15 = sulfamethazine; 16 = sulfamethoxypyridazine; 17 = sulfachloropyridazine; 18 = sulfamethoxazole; 19 = sulfisoxazole; 20 = phthalyl sulfathiazole; 21 = sulfabenzamide; 22 = sulfisomindine; 23 = sulfadimethoxine; 24 = sulfaquinoxaline.

follows: Roos[15] (μBondapak C_{18}, 10 μm, 300 × 3.9 mm), 13 peaks separated in 50 min, peak widths 0.8 at 10 min, 1.6 at 20 min and 2.9 at 40 min. This study (Micropak MCH-10 C_{18}, 10 μm, 300 × 1.0 mm), for the analytes in common, 16 peaks separated in 50 min, peak widths 0.57 at 10 min, 1.2 at 20 min and 2.0 at 40 min. The same mobile phase was used in each case and the elution order was similar.

Fig. 2 is the chromatogram obtained for the isocratic analysis of the mixture of 22 sulfonamides and 3 DHFR standards on the 30-cm 3-μm phase. Twenty peaks were obtained in 107 min. The sulfapyridine (11) peak is broadened as was expected from its $pK_{a,1}$ and the work of Rotsch *et al.*[21]. The isomindine peak (22) is similarly broadened, badly of course due to its late elution. Sulfamoxole (12) hydrolyses in aqueous solutions to form the hydrolysis products 1 (3) and 2 (6). Phthalyl sulfathiazole (20) also slowly hydrolyses to sulfathiazole (8).

Fig. 3 is the best reproducible gradient separation obtained on the 30-cm 3-μm phase. Table I gives the details of the gradient. For the 22 sulfonamides 22 peaks were observed in 65 min but the third peak is one of the sulfamoxole hydrolysis products (3) and the second peak can be seen to be a fused pair [sulfaguanidine (2) and sulfanilamide (4)]. A considerable number of variations on the stated gradient were

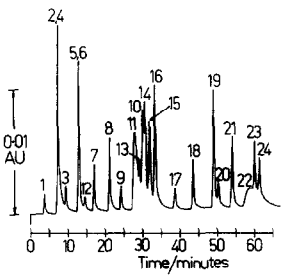

Fig. 3. Gradient analysis of the standard mixture of 25 ng of each of the 22 sulfonamides and 3 DHFRs on the 30-cm capillary column. Gradient conditions are given in Table I. Identifications as in Fig. 2.

TABLE I

GRADIENT CONDITIONS

	Time (min)				
	0	*20*	*25*	*55*	*90*
%A (water)	60	–	–	–	–
%B (acetonitrile)	–	–	–	15	60
%C (acetic acid–acetonitrile–water, 1:12.5:86.5)	40	100	100	85	40
Total acetonitrile (%)	5	12.5	12.5	25.6	65

tried, initially to improve the resolution of the 6 peaks eluted between 27 and 33 min. Weaker or stronger initial gradients and/or initial solvent strengths invariably lead to loss of resolution in this region, frequently lead to fusion of the pair eluted around 50 min [sulfisoxazole (19) and phthalyl sulfathiazole (20)] and often resulted in poorer peak shape and/or worse positioning of the highly mobile sulfisomindine peak.

The introduction of methanol at various times was universally unhelpful. In view of these experiences, very weak gradient and isocratic steps were inserted at the start in an attempt to separate sulfaguanidine and sulfanilamide without disturbing the rest of the separation. These compounds were only resolved under extremely weak conditions and then at great cost of increased analysis time and serious loss of resolution in those vulnerable areas of the chromatogram already mentioned.

Diaveridine, trimethoprim and pyrimethamine are absent from both Fig. 2 (isocratic) and Fig. 3 (gradient) in spite of being present in the standard mixture. Spiking the mixture with several times the initial 25 ng or running concentrated samples of the individual DHFRs failed to yield identifiable peaks even when run in mobile phases containing only highest purity bottled water and chromatographed under strong eluting conditions (80% acetonitrile) for extended periods. There is no change in the absorption band of trimethoprim near 270 nm between 5 and 60% acetonitrile. Irreversible adsorption on the column is indicated.

To investigate the possibility of chelation of the DHFRs with metal impurities in the silica, EDTA was added to the mobile phase. With EDTA added to reservoir C at $5 \cdot 10^{-5}$ mol l^{-1} (mobile phase as for time 0, Table I; 5% acetonitrile overall), a peak emerged at the solvent front for each of the DHFRs. In attempt to achieve retention, the EDTA concentration was decreased. When no peak was observed, a systematic study of the effect of EDTA concentration yielded the following results for trimethoprim. At sufficiently low concentrations of EDTA, there was no effect observed. At sufficiently high concentrations of EDTA, trimethoprim was eluted at the solvent front. At intermediate concentrations of EDTA, fractions of the trimethoprim were eluted, but again at the solvent front.

A further indication of the complicity of the metal impurities in the Phase Sep silica is given by the elution of the DHFRs from the alternate stationary phase prepared from the metal-free Vydac silica.

EDTA appears to play a complex role in the elution of the DHFRs from the MicroPak SP C_{18} phase. The dependence upon EDTA concentration is consistent

with a competitive chelating function, however, the amount of DHFR kept in solution by EDTA blocking some or all of the metal-binding sites seems to be prevented from interacting with the C_{18} hydrocarbon part of the stationary phase. This implies the formation of a polar, lipophobic complex. At pH 2.75, EDTA is present in aqueous solution in about equal parts of the doubly (H_2Y^{2-}) and singly charged (H_3Y^{1-}) forms with 5% of the uncharged form (H_4Y) (ref. 28). No pK_a data could be located for the DHFRs, but a consideration[29] of the tabulated effects of amino and alkyl substituents on the pK_a values of pyridine and pyridazine[30] leads to an estimate of 7.5 for the $pK_{a,2}$ of the ring nitrogens, indicating that the DHFRs have a single positive charge at pH 2.75. The formation of a DHFR–EDTA (1:1) hydrophilic complex with a single net negative charge therefore seems possible.

Fig. 4 is the chromatogram obtained for the isocratic analysis of the 22 sulfonamides on the 60-cm capillary column using the same stationary phase, mobile phase, flow-rate and sample as on the 30-cm column (Fig. 2). Twenty-one peaks were obtained for the 22 sulfonamides in 260 min. There is only one co-elution of sulfas [sulfameter (13) and sulfamethizole (14) at 44 min]. Sulfaguanidine (2) and sulfanilamide (4) at 12–13 min are now largely resolved and the crowded group of peaks centered on 17 min in Fig. 2 and 30 min in Fig. 3 (31–49 min in Fig. 4) are also further separated: sulfapyridine (11) and sulfamoxole (12) are better resolved than previously and are separated from the following three peaks to which they were previously fused.

As there was a factor of 2 difference in length, the increased resolving power of the longer column was expected to be 1.41. Table II gives the resolution (R_s) of all pairs of peaks that are at least partially separated, up to the times after which baseline separation is achieved for all compounds on both columns. Retention times were taken directly from the printout from the data station for Figs. 2 and 4, as were the peak widths at half-height for Fig. 2 (30-cm column). In the case of the 60-cm column, peak widths at the half-height were taken from chromatograms of more concentrated samples where the assignments were unambiguous. The ratios of $R_s(60)/R_s(30)$ are distributed about 1.41 as predicted and the mean of all 11 values is 1.42. Whilst this result would be self-evident for an homologous series, and perhaps for other series in which the molecular structure varied systematically, inspection of the structures[19,25,31] of the sulfonamides reveals that the R and R′ substituents vary in all of shape, size and polarity so that the 22 make up a set not dissimilar to the phenylthiohydatoin amino acids. It is impossible to predict much of the detail of the elution order simply on the basis of structure. Also, it was observed in the gradient work on the 30-cm column that either stronger or weaker conditions of elution lead to rapid deterioration of the separation in the most congested region of the chromato-

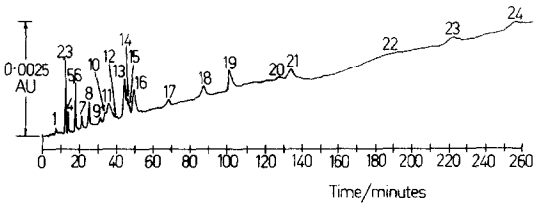

Fig. 4. Isocratic analysis of the standard mixture of 25 ng of each of the 22 sulfonamides and 3 DHFRs on the 60-cm capillary column. Identifications as in Fig. 2.

TABLE II

COMPARISON OF RESOLUTION BETWEEN 30- AND 60-cm CAPILLARY COLUMNS

t_R = Retention time (min); R_s = resolution; (30) and (60) = 30- and 60-cm columns, respectively; HP = hydrolysis product.

No.	*Drug*	$t_R(30)$	$R_s(30)$	$t_R(60)$	$R_s(60)$	$R_s(60)/R_s(30)$
1	Sulfanilic acid	3.672	2.660	7.783	5.249	1.974
2	Sulfaguanidine	5.667		12.945		
3	Sulfamoxole, HP1	5.720		12.945		
4	Sulfanilamide	5.774	2.275	13.763	3.691	1.622
5	Sulfacetamide	7.500		17.838		
6	Sulfamoxole, HP2	7.500	1.995	17.838	2.600	1.303
7	Sulfadiazine	9.202	1.571	21.287	2.345	1.493
8	Sulfathiazole	10.662	2.733	25.000	3.212	1.175
9	Sulfamerazine	13.229	0.823	31.114	1.285	1.561
10	Succinyl sulfathiazole	14.001	0.694	33.950	0.729	1.050
11	Sulfapyridine	15.424	0.650	35.722	1.442	2.218
12	Sulfamoxole	16.916	0.905	39.649	1.458	1.611
13	Sulfameter	18.100		44.249		
14	Sulfamethizole	18.140	0.916	45.860	0.260	0.284
15	Sulfamethazine	19.390	0.643	46.710	0.837	1.302
16	Sulfamethoxypyridazine	20.349		49.367		

gram, clearly indicating unsystematic behaviour for that group of analytes. A further indication of the unpredictable nature of chromatographic relationships between the sulfonamides is given by the wide range of values of the $R_s(60)/R_s(30)$ ratio: from 0.284 to 2.218. These factors highlight the advantage of longer columns and higher total available plates in difficult liquid chromatographic separations of complex mixtures. Gradient work on the 60 cm column would reduce the analysis time by at least a factor of 2 and would have good potential for the separation of all pairs of sulfonamides.

However, the attenuation in Fig. 4 is only half of that in Fig. 2. As the sample is the same but the retention is approximately doubled, we would therefore have expected peak heights and areas to have been the same. Inspection of the two chromatograms shows a huge deficiency in Fig. 4. Peak areas losses are between 80 and 96%. Doubling the exposure times of the sulfonamides —and to a fresh stationary phase— has drastically exacerbated any losses that must have occurred on the shorter column. Hence a retrospective estimate was made of losses on the 30-cm capillary column. Because the microbore and capillary work were done at the same linear flow-rate (within experimental error of the micro flow meter), the peak volumes will be proportional of the square of the column internal diameters. Hence the peak areas obtained on the 1 mm, C_{18}-10 metal-free stationary phase have been scaled-up to compare with those actually obtained on the same length, 0.35 mm, C_{18}-3 Phase Sep column. Losses are thus estimated between 46 and 73% with a mean of (59 $\pm$ 9)%.

In spite of the observed losses of sulfonamides when small amounts are chromtographed, I believe the above results will be of interest in showing the potential for better separations by the use of narrow bore columns. However, I have neither had the need nor the opportunity to investigate the above problems further.

A stationary phase of similar particle size, surface area and carbon loading but

free from metal contamination may provide a good basis for trace analysis of the sulfonamides and DHFRs.

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