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HIGH-PERFORMANCE ION-PAIR PARTITION CHROMATOGRAPHY OF SULFA DRUGS

STUDY AND OPTIMIZATION OF CHEMICAL PARAMETERS

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SUMMARY

High-performance ion-pair partition chromatography is shown to be a versatile, efficient method for separating sulfonamides. For a group of fourteen sulfa drugs varying widely in pK_A and hydrophobicity, the effect of mobile phase composition, counterion composition, pH, and ionic strength on their ion-pair partition chromatographic separation using tetrabutylammonium as the counterion and *n*-butanol–*n*-heptane as the mobile phase is shown. Wide variation in k' and α is possible by changing these parameters. Silica columns coated with buffered aqueous solutions of tetrabutylammonium sulfate resulted in efficiencies of 4000–6000 theoretical plates per 25 cm. These columns are stable for long periods of time, and can be stripped and re-used in the adsorption mode with little or no loss in efficiency. Several chromatograms are presented in order to illustrate the performance of ion-pair partition chromatography.

INTRODUCTION

In previous papers[1,2] the potential of high-performance ion-pair partition chromatography for the separation of ionizable organic compounds has been demonstrated. The resolution of closely related solutes was achieved by the combination of the inherent high selectivity of the ion-pair partition process, coupled with the high efficiency of modern liquid chromatographic columns. The work has drawn much from the extensive literature on ion-pair extraction (see, *e.g.*, ref. 3). Ion-pair partition chromatography using a variety of column packings has been applied successfully by other researchers as well, using both normal[4–7] and reversed-phase[8,9] systems. A related technique using liquid ion exchangers in the stationary phase has also been utilized[5,10].

In order to illustrate further the potential of the method, this paper will present a fundamental study of the parameters influencing retention and selectivity in the high-performance ion-pair partition chromatography of a related series of sulfon-

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TABLE I
SULFA DRUGS USED IN THIS STUDY

R_1—NH—⟨◯⟩—SO_2—NH—R_2

No.	*Name*	R_1	R_2	pK_A*
1	Phthalyl sulfathiazole	O, C, CO_2H	S, N	—
2	Sulfabenzamide	H	O, C	4.6
3	Sulfisoxazole	H	O, N, H_3C, CCH_3	4.9
4	Sulfacetamide	H	O, C, CH_3	5.4
5	Sulfadimethoxine	H	N, OCH_3, N, OCH_3	6.0
6	Sulfachloropyridazine	H	Cl, N–N	6.1
7	Sulfadiazine	H	N, N	6.4
8	Sulfaquinoxaline	H	N, N	6.5**
9	Sulfamerazine	H	N, CH_3, N	6.9
10	Sulfamethoxypyridazine	H	OCH_3, N–N	7.2
11	Sulfathiazole	H	S, N	7.2
12	Sulfamethazine	H	N, CH_3, N, CH_3	7.6
13	Sulfapyridine	H	N	8.4
14	Sulfanilamide	H	–H	10.4

* Median of literature values [33–36].
** Estimated on the basis of retention data.

amides. The compounds studied are listed in Table I. All have a common acidic N–H linkage adjacent to the sulfonyl group. Therefore, a basic pH and a cationic counterion (tetrabutylammonium ion, TBA^+) were used to carry out ion-pair partition. It should be noted that these molecules differ widely in the acid dissociation constants and the hydrophobicity of the organic side groups, and hence selectivity in ion-pair partition should be particularly sensitive to chromatographic conditions.

Beyond their use as an interesting class of substances for study, sulfa drugs represent an important analytical problem in terms of trace analysis in animal tissues (0.1 ppm) and milk (0.01 ppm)[11]. Current standard methods for these compounds[12], as well as a variety of modifications (see, *e.g.*, refs. 13–17) generally involve thin-layer or paper chromatography followed by colorimetric detection using the Bratton–Marshall procedure. In addition, both gas chromatography[18–20] and column chromatography[21] have been proposed as alternate separation methods. High-performance liquid chromatography (HPLC) offers advantages over these methods from the high selectivity and efficiency that can be achieved within a single column. In addition, very low levels of sulfa drugs can be conveniently detected by means of a UV detector (ε at 254 nm *ca.* 10^4). Several papers describing HPLC ion-exchange separations of sulfonamides have recently appeared[22,23], but the fine tuning possible with ion-pair partition HPLC as exemplified in this paper makes this method a good choice for this separation problem.

THEORY

If partition between the two phases is the only significant retention mechanism, then the capacity factor k' will be given by

$$k' = D\, V_w / V_o \tag{1}$$

where D is the bulk liquid distribution ratio and V_w and V_o are the volumes of aqueous (stationary) phase and organic (mobile) phase, respectively. The equilibria associated with ion-pair partition are generally well understood (see, *e.g.*, ref. 3). Before equilibrium expressions for D can be derived, several assumptions must be made, *viz.*

(1) Only 1:1 ion pairs form.

(2) The equilibrium concentration of ion pairs in the aqueous phase is very low relative to that of free ions.

(3) Only neutral species are partitioned into the organic phase.

(4) Ion-pair formation with species other than the counterion is negligible.

(5) Dissociation or dimerization of the ion pairs in the mobile phase is negligible.

(6) Liquid–liquid bulk equilibrium is the only significant chromatographic retention mechanism.

Composition of phases

The aqueous stationary phase used in this work consists of a buffered solution of tetrabutylammonium bisulfate ($TBAHSO_4$). Since the pH of this phase is always greater than six, the predominant species present are TBA^+ and SO_4^{2-}. The organic mobile phase consists of *n*-heptane containing various amounts of *n*-butanol (BuOH)

to increase the solubility of ion pairs in this phase. When these two phases are equilibrated, small amounts of water, $TBAHSO_4$, H_2SO_4, and various ion-paired buffer species will be transferred to the organic phase, and small amounts of BuOH and *n*-heptane will be transferred to the aqueous phase. The extent of transfer was assessed in several ways. pH measurements before and after equilibration showed that the pH of the stationary phase remained constant. In addition, gas chromatographic analysis of the two phases after equilibration was carried out. The aqueous phase was found to contain between 4 and 5% (w/w) BuOH and a negligible amount of *n*-heptane under a wide range of conditions. On the other hand, the amount of water extracted into the organic phase depended on the butanol concentration in that phase, ranging from 1.5% (w/w) with 25% (v/v) butanol to 5.0% (w/w) with 50% (v/v) butanol in heptane. In all cases the resulting composition changes in the major components of the two phases are small and for simplicity will be neglected as long as the phase ration are near 1:1, which is the case for the chromatographic columns used in this work.

Sulfonamide equilibria

When a sulfonamide (HS) is added to the two-phase system, the predominant species present will be S^- in the aqueous phase and the ion pair (TBA^+, S^-) in the organic phase, and/or HS in both phases, depending on the pH of the stationary phase. As shown previously[2], (TBA^+, S^-) will be "solvated" by the butanol to form (TBA^+, S^-)·*n*BuOH, where *n* was found to be the same for all sulfonamide ion pairs. It must be emphasized that *n* may not be a conventional solvation number, but may merely indicate the number of BuOH molecules associated with (TBA^+, S^-) in the phase. This point will be discussed further below. It is reasonable to expect that any HS present in the organic phase will also be "solvated" to form HS·*m*BuOH. The apparent solvation number of HS for each sulfonamide has not been determined (however, see discussion of Fig. 3). Nevertheless, we will include this form of HS in the expressions below for completeness.

The overall distribution constant for a sulfonamide between the two phases can thus be written as

$$D = \frac{[HS]_w + [S^-]_w}{[HS \cdot m\mathrm{BuOH}]_0 + [(TBA^+, S^-) \cdot n\mathrm{BuOH}]_0} \tag{2}$$

In the aqueous phase, $[HS]_w$ and $[S^-]_w$ are related through the ionization constant K_A

$$K_A = \frac{[H^+]_w [S^-]_w}{[HS]_w} \tag{3}$$

Also, equilibrium constants can be written for the partition of (TBA^+, S^-) and HS

$$(TBA^+, S^-) \cdot n\mathrm{BuOH} \rightleftharpoons TBA^+_w + S^-_w + n\mathrm{BuOH}$$

$$K_1 = \frac{[TBA^+]_w [S^-]_w [\mathrm{BuOH}]^n}{[(TBA^+, S^-) \cdot n\mathrm{BuOH}]_0} \tag{4}$$

$$HS \cdot m\mathrm{BuOH} \rightleftharpoons HS_w + m\mathrm{BuOH}$$

$$K_2 = \frac{[HS]_w [BuOH]^m}{[HS \cdot mBuOH]_o} \tag{5}$$

In general, with TBA^+ as a counterion for acids, ion-pair partition equilibrium constants are greater than undissociated acid partition equilibrium constants. Thus, unless $m \ll n$ (which is unlikely), we would expect $K_1 > K_2$. Substituting from eqns. 3, 4, and 5 and factoring out $[S^-]_w$ gives

$$D = \frac{1 + ([H^+]_w/K_A)}{([TBA^+]_w [BuOH]^n/K_1) + ([H^+]_w [BuOH]^m/K_2 K_A)} \tag{6}$$

Eqn. 6 relates D (and K) to a number of parameters which can be varied to control retention and selectivity. The equation can be simplified by considering two limiting cases, *viz.*

(A) $pK_A \ll$ pH of stationary phase. In this case the sulfonamide will be completely dissociated in the stationary phase, and ion-pair partition will be the dominant retention mechanism. Since $[H^+]_w/K_A \ll 1$,

$$D_1 = \frac{K_1}{[TBA^+]_w [BuOH]^n} \tag{6A}$$

(B) $pK_A \gg$ pH of stationary phase. In this case the sulfonamide will be essentially undissociated in the stationary phase, and partition of HS will be the dominant retention mechanism. Since $[H^+]_w/K_A \gg 1$, eqn. 5 will simplify to

$$D_2 = \frac{K_2}{[BuOH]^m} \tag{6B}$$

EXPERIMENTAL

Chromatographic apparatus

A component liquid chromatograph system was used for all experiments. This consisted of a Waters Associates M6000 pump and U6K injector, a Laboratory Data Control 1206 V detector (254 nm), and a Texas Instruments Redi-Riter strip chart recorder. In order to minimize stationary phase bleeding as well as baseline noise and drift, all components except the recorder were confined within an air bath box at a temperature of 27 ± 0.5° (2–3° above ambient). A 150-W light bulb served as the heating element, which was controlled by a Princo T-681D relay and Haake mercury contact thermostat (−35 to +105°). A continuously operating fan was used as an air circulator. It is estimated that the short-term temperature variation of the mobile and stationary phases in this system was held constant to ± 0.2°. (More precise analysis would require a water-jacketed precolumn/column system.)

Preparation and equilibration of phases

Stationary phase. A known mass of $TBAHSO_4$ and/or Na_2SO_4 (to control ionic strength) was added to 500 ml of a solution of NaH_2PO_4 (pH 6.8 or 7.4) or H_3BO_3 (pH 8.5 or 9.2) and the resulting solution titrated with 0.5 M NaOH until the desired pH (± 0.05 unit as measured with a standard pH meter) was obtained.

Mobile phase. A precisely measured volume of BuOH was added to a 500-ml volumetric flask and diluted to the mark with *n*-heptane.

Equilibration. The mobile and stationary phases were mixed in a 1-l flask and stirred at 27° in the air bath for several hours to ensure complete phase equilibration. The two phases were allowed to separate, and the aqueous layer which contained an equilibrium concentration of the organic solvent was then used for column coating.

Column preparation

Home-made high-performance columns were employed in all the experiments. These consisted of Analabs 1/4-in. precision bore tubing (25 cm × 3.2 mm I.D.) with bored-out Swagelok end fittings and Altex 2-μm frits. The column packing used was E. Merck LiChrospher SI-100 spherical silica (10-μm diameter, 250 m^2/g). The columns were packed using a balanced-density slurry technique[24] slightly different from that reported by Majors[25].

Column efficiency was first evaluated in the adsorption mode, using 1% isopropanol–*n*-heptane as a mobile phase and benzyl alcohols as solutes. In addition, the nonsorbed time (t_0) of an unretained solute (benzene) was carefully measured for use ultimately in obtaining the stationary phase per cent loading determinations.

Coating and stripping procedures

A direct injection technique[26] was used to heavily load the columns. All coating was carried out at 27° in the air bath. The mobile phase reservoir used in this and subsequent steps contained both the aqueous and the organic phase, which were stirred gently to ensure continued equilibration. First the column was washed by pumping at least 50 ml of mobile phase through it. Then three injections of 2.0 ml each of stationary phase were made at 5-min intervals with a flow-rate of 1.5 ml/min. After excess stationary phase had completely eluted from the column (as indicated by the absence of an emulsion at the column outlet), the column was conditioned at 2.5 ml/min in the recycle mode for at least 3 h. After conditioning the retention time of benzene was obtained at the same flow-rate as in the adsorption measurements. Per cent loading was determined from Δt_0 before and after coating and the known weight of the packing (0.67 ± 0.01 g, determined by unpacking several columns and weighing the dried column packing). The loading of columns prepared in this manner was found to be 52 ± 2% (w/w).

For conversion back to the adsorption mode, the columns were successively washed with at least 50 ml each of water, methanol, methylene chloride, and *n*-heptane, and then re-equilibrated with 1% isopropanol–*n*-heptane mobile phase. For recoating with aqueous stationary phase the columns were washed as above except that the *n*-heptane step was omitted, and the column was re-equilibrated with butanol–*n*-heptane mobile phase before recoating.

Static determination of distribution ratios

The distribution ratios were determined in the manner previously reported[2]. A small quantity of solute was dissolved in 1 ml of the aqueous phase and then extracted into 40 ml of organic phase. The absorbance of the organic phase, which contained the solute ($\lambda_{max.} \approx$ 270 nm), was measured using a Cary 118 spectrophotometer. A known volume of this solution was then carefully shaken with a known

volume of the aqueous phase in a thermostatted bath, at 27°. The absorbance in the organic phase was again measured, and the distribution constant calculated.

Composition of phases and pK_A values

In order to determine the water content in the mobile phase, the per cent of water, butanol and heptane for various mobile phases (after equilibration with given stationary phases) were measured. A gas chromatograph (Perkin-Elmer F 11 GC) with a thermal conductivity detector and a Porapak Q column, at 215°, were used. Per cent by weight was calculated from the peak area ratios using thermal conductivity correction factors[27].

Chemicals

The sources of the sulfa drugs have been previously listed[2]. Organic solvents were obtained from Burdick & Jackson (Muskegon, Mich., U.S.A.), and tetrabutylammonium hydrogen sulfate from Sigma (St. Louis, Mo., U.S.A.). Inorganic salts of analytical reagent grade were obtained from various sources. All chemicals were used without further purification.

RESULTS AND DISCUSSION

Chromatographic performance

Prior to coating with stationary phase, the columns used in this work had efficiencies ranging between 10,000 and 12,000 theoretical plates per 25 cm at the optimum velocity of *ca.* 0.08 cm/sec with *n*-heptane as a mobile phase. After coating with stationary phase, the plate counts of the columns decreased; however, good efficiencies were still achieved, as indicated in Fig. 1. At reasonable velocities (*ca.* 0.1–0.2 cm/sec) and $k' > 1$ approximately 5000 theoretical plates are obtained for a 25-cm column. Much of the decrease in efficiency relative to adsorption can be

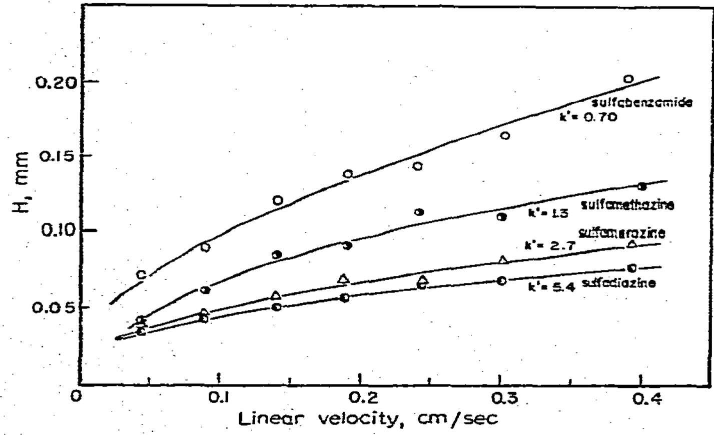

Fig. 1. H *vs.* linear velocity v for several sulfonamides. Mobile phase, 40% (v/v) *n*-butanol in *n*-heptane; stationary phase, 0.1 M $TBAHSO_4$ in 0.1 M borate buffer, pH 8.4.

attributed to mobile phase viscosity differences (*i.e.*, the viscosity of *n*-heptane is *ca.* 0.4 cP, the viscosity of *n*-heptane with 25% *n*-butanol is *ca.* 1.2 cP). Peak symmetry in ion-pair partition chromatography is also very good, as can be seen from Figs. 5–7.

The high efficiencies of these columns in the adsorption mode are not significantly affected by their use for liquid–liquid chromatography. When the stationary phase is stripped off and the column re-evaluated for adsorption, the high efficiencies are again found (*i.e.*, *ca.* 10,000 plates per 25 cm). Thus, a given column can be used for both adsorption and ion-pair partition interchangeably.

Stability and reproducibility

To be useful for routine analysis, ion-pair partition columns must obviously be stable and reproducible. The effective lifetime of any liquid–liquid chromatographic column depends on many factors such as: per cent loading, mobile phase flow-rate, degree of miscibility of the phases, etc. With careful preparation and use, there was no significant change in the retention or efficiency characteristics for times greater than one month. (We have not studied in detail the long-term stability beyond this time period.) It should be emphasized that if and when column performance has deteriorated beyond an acceptable limit, it is relatively simple to change to new mobile and stationary phases by stripping and recoating, as described in Experimental. The reproducibility of this procedure is very good, as can be seen in Table II. A previously coated column was stripped and recoated three times, each time using new batches of mobile and stationary phase. It can be seen that k' is reproduced within 2–4%, and α within 1–2%.

TABLE II

REPRODUCIBILITY OF COLUMN CONDITIONS

Stationary phase, 0.1 *M* $TBAHSO_4$ in 0.1 *M* borate buffer, pH 8.4. Mobile phase, 25% (v/v) *n*-butanol in *n*-heptane.

Sulfonamide No.	*k′ after recoating**			α**	δ (%)***
	Experiment No. 1	*Experiment No. 2*	*Experiment No. 3*		
1	0.60	0.69	0.80	0.22	13
2 and 8§	2.03	2.08	2.08	0.65	3
3	3.22	3.19	3.10	1.00	—
6	3.38	3.43	3.35	1.07	2
11	3.77	3.95	3.80	1.21	3
5	6.18	6.10	5.93	1.91	2
12	7.79	7.74	7.33	2.41	1
10	10.8	10.6	10.1	3.31	1
13	13.5	14.2	14.1	4.26	4
9	18.6	18.1	17.1	5.66	2
7	41.0	39.6	37.4	12.4	2
4	48.8	46.4	44.3	14.6	2
14	73.5	71.8	66.3	22.2	2

* See text for details.

** Average for the three experiments.

*** Relative standard deviation of α (estimated as 1.25 × average deviation).

§ Nos. 2 and 8 are eluted together under these conditions.

Static versus chromatographic distribution ratios

If liquid–liquid partition is the only retention mechanism, then statically measured distribution ratios should agree with those obtained chromatographically. Since V_s/V_m is difficult to measure precisely, it is better to compare relative values of D and k'. Given in Table III are static and chromatographic relative constants for several sulfonamides at two different pH values. The agreement between the two sets of constants is quite good, considering the $\pm$ 2% reproducibility of the k' values and the difficulty of obtaining the higher D values due to the low measured concentrations involved. Therefore, we can conclude that in this system the only retention mechanism is bulk partition, in agreement with our previous work[2].

TABLE III

COMPARISON OF STATIC AND CHROMATOGRAPHIC DISTRIBUTION RATIOS

Organic phase, 25% (v/v) *n*-butanol in *n*-heptane; aqueous phase, 0.1 M TBA^+ in 0.1 M buffer at the pH specified.

Solute	*Relative D values*			
	pH 7.4		*pH 8.4*	
	Static	*Chrom.*	*Static*	*Chrom.*
Sulfachloropyridazine	1.00	1.00	1.00	1.00
Sulfadimethoxine	1.49	1.68	1.76	1.82
Sulfamethazine	2.84	2.98	2.38	2.31
Sulfamerazine	5.50	5.45	5.95	5.53
Sulfapyridine	6.39	6.27	3.98	3.98
Sulfanilamide	23.7	21.9	19.8	21.6

Study of chemical variables —presence or absence of TBA^+ counterion

In the presence of TBA^+, D can be written in terms of eqn. 6. In the absence of TBA^+, for sulfonamides whose $pK_A \ll$ pH of the stationary phase, k' is expected to be quite large. On the other hand, k' for undissociated sulfonamides should be the same at a given pH whether or not TBA^+ is present. Evidence supporting these conclusions is given in Table IV. It can be seen that for sulfonamides whose pK_A is

TABLE IV

COMPARISON OF RETENTION WITH AND WITHOUT COUNTERION IN THE STATIONARY PHASE

Solute	pK_A	*k' values**	
		*Without counterion***	*With counterion****
Sulfisoxazole	4.9	>100	2.86
Sulfachloropyridazine	6.1	>100	3.07
Sulfamethoxypyridazine	7.2	80	9.90
Sulfamethazine	7.6	37	6.76
Sulfapyridine	8.4	20	9.30
Sulfanilamide	10.4	45	74.2

* Mobile phase, 25% (v/v) *n*-butanol in *n*-heptane.
** Stationary phase, 0.1 M Na_2SO_4 in 0.1 M buffer, pH 8.4.
*** Stationary phase, 0.1 $TBAHSO_4$ in 0.1 M buffer, pH 8.4.

substantially less than the pH of the stationary phase, k' decreases by a factor of more than 30 when TBA^+ is added as a counterion. The decrease is smaller for partially ionized (*i.e.*, less than 99% dissociated) sulfonamides, as would be expected, the magnitude of the decrease being approximately inversely proportional to the pK_A of the compound. The increase in k' for sulfanilamide, which is unionized at a pH of 8.4, can be attributed to the change from a salting-out system (0.1 *M* Na_2SO_4) to a salting-in system (0.1 *M* $TBAHSO_4$) for hydrophobic molecules[28].

Variation in TBA^+ concentration

Having demonstrated the effectiveness of TBA^+ as a counterion, the influence of varying the concentration of the counterion in the stationary phase can be considered. For fully ionized sulfonamides eqn. 6A indicates that D (or k') should be inversely proportional to $[TBA^+]_w$. For undissociated sulfonamides k' should be independent of $[TBA^+]_w$.

Plots of log k' *vs.* log $(1/[TBA^+]_w)$ for several sulfonamides are presented in Fig. 2. As predicted from eqn. 6A, for ionized compounds (Nos. 6, 9, and 12) the plots are linear and parallel. Thus, for completely ionized sulfa drugs relative retention is independent of TBA^+ concentration. Identical behavior is shown at this pH for all other sulfonamides except sulfapyridine and sulfanilamide. It is seen that the former compound (No. 13) has a lower slope since at a pH of 8.5 it is only 50% ionized. Thus, as expected, the effect on its retention of increasing $[TBA^+]_w$ is not as great as in the case of fully ionized sulfa drugs. The slope of the plot for sulfanilamide (No. 14) is slightly negative; this can be attributed to salting-in by TBA^+, as discussed above.

The results given in Fig. 2 indicate that while selectivity in the ion-pair partition liquid chromatographic separation of ionized sulfonamides is independent of the

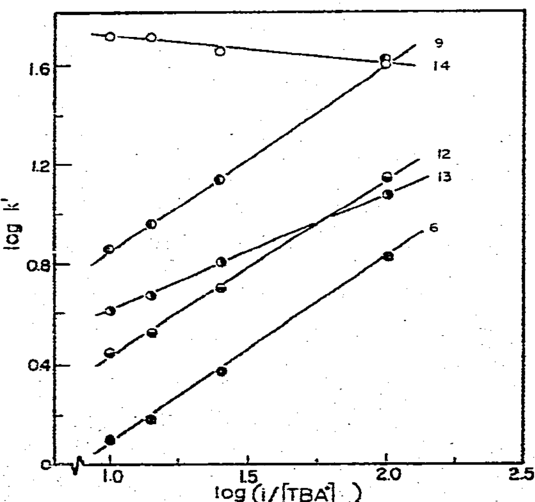

Fig. 2. Log k' *vs.* log $(1/[TBA^+]_w)$ for several sulfonamides. Mobile phase, 25% (v/v) *n*-butanol in *n*-heptane; stationary phase, $TBAHSO_4$ in 0.1 *M* borate buffer, pH 8.5 and $\mu = 1.1$ (adjusted with Na_2SO_4). Compound numbers refer to Table I.

counterion concentration, for unionized or partially ionized sulfonamides relative retention will vary, and sometimes reversals in retention order will occur. Thus, in general it is best to choose a counterion concentration and pH which give good selectivity for the solute mixture of interest, and then adjust k' via changes in the mobile phase composition (see the next section).

Variation in butanol concentration

For all sulfonamides, at constant pH and $[TBA^+]_w$, according to eqns. 6A and 6B k' should decrease as [BuOH] increases, and plots of log k' *vs.* log [BuOH] should be linear. Such plots for several sulfonamides are shown in Fig. 3. As expected, they are linear, and except for sulfanilamide (which is unionized) all lines have the same slope: —3.40. Thus the "effective" solvation number of the ion pairs in the organic phase is 3.40, in agreement with that found in our previous work at a different pH (ref. 2). The slope for sulfanilamide is 2.55, indicating a lower degree of solvation.

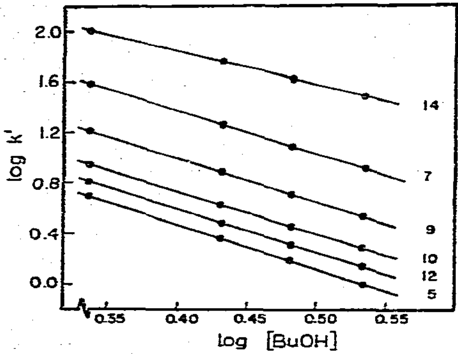

Fig. 3. Log k' *vs.* log [BuOH] for several sulfonamides. Mobile phase, *n*-butanol in *n*-heptane; stationary phase, 0.1 *M* $TBAHSO_4$ and 0.25 *M* Na_2SO_4 in 0.25 *M* borate buffer, pH 8.5. Compound numbers refer to Table I.

As discussed in Theory, these solvation numbers must be interpreted with caution. For example, butanol is known to self-associate in both organic[29] and aqueous[30] solvent systems, and so the effective solvation numbers obtained will reflect these phenomena. Furthermore, water molecules are most probably also associated with the protonated or ion-paired sulfonamide and/or the solvating butanol in the organic phase. Thus the real species dissolved in the organic phase may be quite complex. Chromatographically, the exact nature of the species is not as important as the measured solvation number, since the latter indicates that care must be exercised in the preparation of the phases if good reproducibility is to be obtained. On the other hand, the high constant exponent indicates that changes in k' (at constant relative retention) are easily achieved through variation of the mobile phase composition.

Variation in pH

In the stationary phase pH region where pK_A is either $\ll$ or $\gg$ pH, D (or k') should be independent of pH. On the other hand, for sulfonamides whose pK_A falls

in the region of a pH change, as the pH of the stationary phase is increased from a value less than to a value greater than the pK_A, the retention mechanism will change from partition of HS to partition of (TBA^+, S^-). Plots of k' or α *vs.* pH in this region should resemble a titration curve. Similar behavior has been observed in ion exchange[31] and reversed-phase liquid chromatography[32].

Plots of relative retention *vs.* pH are given in Fig. 4 for several sulfonamides. For the three compounds whose pK_A values are less than 6.0, α is independent of pH, as expected, since the partition mechanism involves exclusively the ion-pair distribution process. The behavior of sulfapyridine (No. 13), sulfamethazine (No. 12), and sulfathiazole (No. 11) is also as expected, since the pK_A values of these compounds fall in the pH range studied. As pH is increased from a value less than to a value greater than the pK_A, α (and k') decrease, and the apparent inflection points of the curves (8.5, 7.5, and 7.0) correspond roughly to the pK_A values of the compounds. This type of behavior indicates the great control one has over selectivity in the ion-pair partition chromatographic separation of acidic or basic compounds. Significant decreases or increases in k' and large changes in α can be achieved by varying the pH of the stationary phase in the vicinity of the pK_A of the compounds being separated.

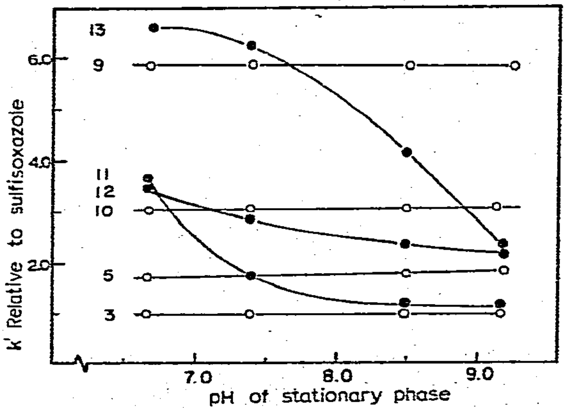

Fig. 4. Relative retention *vs.* pH for several sulfonamides. Mobile phase, 25% (v/v) *n*-butanol in *n*-heptane; stationary phase, 0.1 *M* $TBAHSO_4$ in 0.1 *M* phosphate or borate buffer. Compound numbers refer to Table I.

Ionic strength

The effect of varying ionic strength, μ, on all the equilibria discussed above is difficult to assess precisely. In general, a decrease in k' is expected as ionic strength is increased, and this decrease will be related in a complicated manner to the structure of the sulfonamide, as indicated by the data in Table V. It can be seen that k' decreases by a factor of two to three in all cases when ionic strength is roughly doubled. (Similar changes are observed for all other sulfonamides.) Changes in relative retention are negligible in most cases for compounds which are fully ionized in the stationary phase, but are significant for those which are not and which thus partition as HS. Note, for

TABLE V

EFFECT OF IONIC STRENGTH OF STATIONARY PHASE ON RETENTION

Mobile phase, 25% (v/v) *n*-butanol in *n*-heptane.

Solute	pK_A	*pH 6.7*				*pH 8.5*			
		$\mu = 0.44$*		$\mu = 1.04$**		$\mu = 0.34$***		$\mu = 1.09$§	
		k'	α	k'	α	k'	α	k'	α
Sulfabenzamide	4.6	2.14	1.00	1.46	1.00	1.78	1.00	0.74	1.00
Sulfisoxazole	4.9	3.35	1.57	2.20	1.51	2.86	1.61	1.16	1.57
Sulfadimethoxine	6.0	5.73	2.68	3.75	2.57	5.34	3.00	2.09	2.82
Sulfamethazine	7.6	11.0	5.14	9.18	6.29	6.76	3.80	2.80	3.78
Sulfapyridine	8.4	22.1	10.3	20.0	13.7	9.30	5.22	4.11	5.55
Sulfanilamide	10.4	74.0	34.6	64.2	44.0	74.2	41.7	52.5	71.0

* Stationary phase, 0.10 *M* $TBAHSO_4$ in 0.10 *M* phosphate buffer.
** Stationary phase, 0.10 *M* $TBAHSO_4$ in 0.10 *M* phosphate buffer + 0.20 *M* Na_2SO_4.
*** Stationary phase, 0.10 *M* $TBAHSO_4$ in 0.25 *M* borate buffer.
§ Stationary phase, 0.10 *M* $TBAHSO_4$ in 0.25 *M* borate buffer + 0.25 *M* Na_2SO_4.

example, that at a pH of 6.7 sulfamethazine is substantially unionized, and its relative retention increases as μ increases. On the other hand, at a pH of 8.5 this compound is substantially ionized, and its relative retention does not increase at this pH. The results in Table V thus indicate that salt effects can influence selectivity, depending on the extent of ionization of the sulfa drug.

CHROMATOGRAPHIC SEPARATIONS

With the above results it is relatively easy to choose optimum conditions for rapid, high-resolution separation of a group of sulfonamides. A separation of all fourteen compounds studied is given in Fig. 5. Note that flow programming could

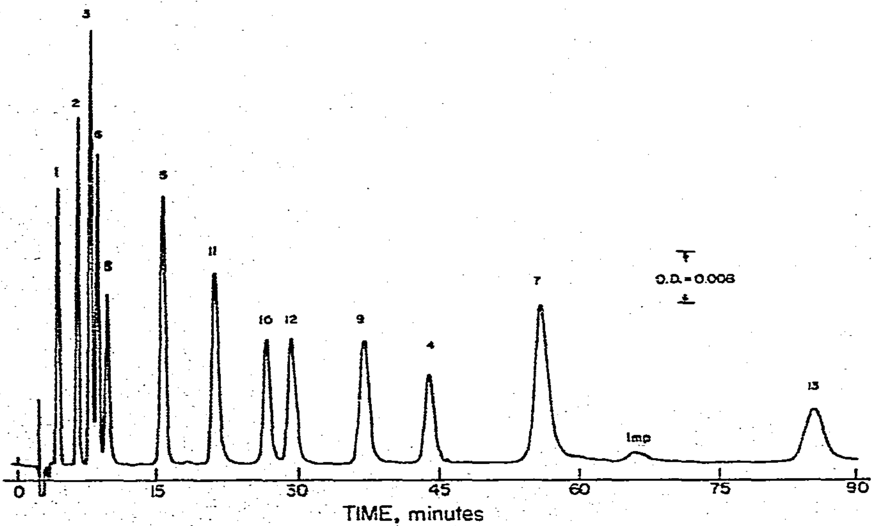

Fig. 5. Separation of sulfonamides. Mobile phase, 25% (v/v) *n*-butanol in *n*-heptane; stationary phase, 0.3 *M* $TBAHSO_4$ in 0.1 *M* phosphate buffer, pH 6.8. Compound numbers refer to Table I.

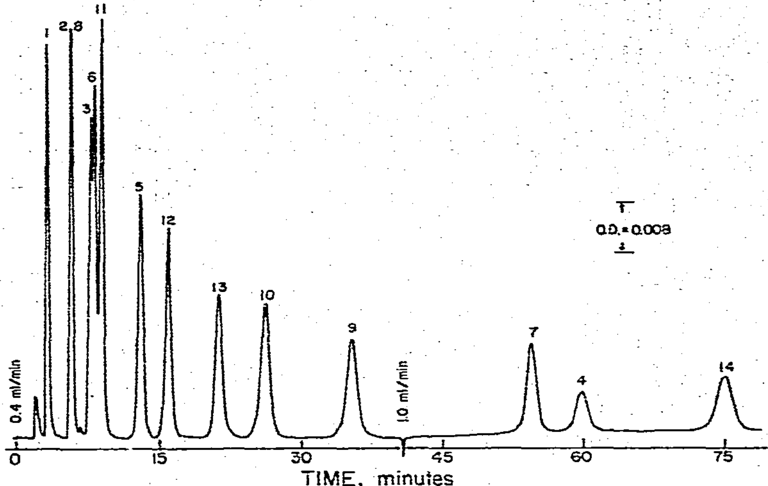

Fig. 6. Separation of sulfonamides. Mobile phase, 25% (v/v) *n*-butanol in *n*-heptane; stationary phase, 0.1 *M* $TBAHSO_4$ in 0.1 *M* borate buffer, pH 8.4. Compound numbers refer to Table I.

have been used during the last part of the separation to decrease the analysis time significantly. Another separation of all fourteen compounds is shown in Fig. 6. The conditions are the same as in Fig. 5, except that the pH has been increased fom 6.8 to 8.4 and $[TBA^+]$ decreased from 0.3 *M* to 0.1 *M*. Note the dramatic decrease in retention for peak 13 (sulfapyridine) as the retention mechanism shifts from that of the free acid to that of an ion pair. For a smaller group of sulfonamides having more similar k' values, even shorter analysis times are possible. Fig. 7 shows the separation of eleven sulfonamides in approximately 10 min.

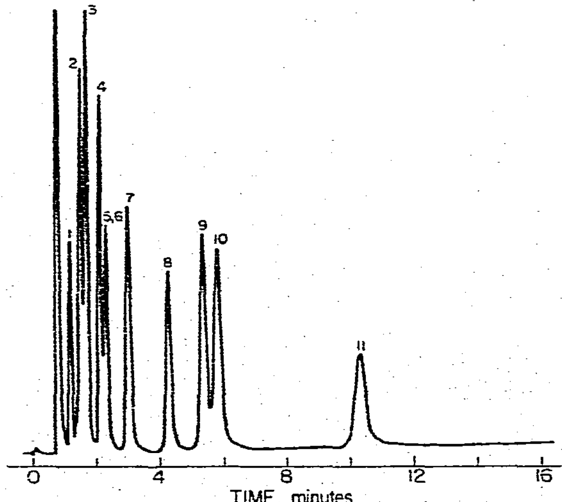

Fig. 7. Rapid separation of sulfonamides. Mobile phase, 25% (v/v) *n*-butanol in *n*-heptane; stationary phase, 0.04 *M* $TBAHSO_4$–0.31 *M* Na_2SO_4 in 0.25 *M* borate buffer, pH 8.5. Compound numbers refer to Table I.

CONCLUSION

Ion-pair partition HPLC has been shown to be a straightforward, easily understandable, flexible, highly reproducible method for the analysis of mixtures of sulfonamides. The large number of parameters available which can be varied to obtain optimum k' and α values allows fine tuning of retention for the separation of a large number of sulfonamides. Work has recently been completed on the development of a method for determining sulfonamides in milk which are present in concentrations of less than one part in 10^9. This will be reported shortly.

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