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ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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CONTENTS

I. INTRODUCTION

It is the purpose of this contribution to present and assess the use of pairing ions in high-performance liquid chromatographic (HPLC) systems in the analysis of ionised solutes both of general chemical interest and, in particular, of pharmaceutical interest. Those constitutional, environmental and operational factors which can affect solute retention in ion-pairing systems will be discussed. Also included is a comprehensive listing of pairing ions currently in use along with the phase systems and solutes examined.

The analysis of non-polar solutes by HPLC is generally a simple task, especially if reversed-phase systems are used. However many compounds of pharmaceutical interest such as drugs, formulation additives (*e.g.* solubilizers, taste maskers), phyto-

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chemicals and synthetic reaction products are ionised or polar species. Additionally, drug metabolism will generally lead to the production in the body of more hydrophilic species, (a process which enables kidney excretion to be performed efficiently), and the formed metabolites will be generally either acidic (or anionic) in character or will be conjugated with some suitable acid such as glycine, etc. By their very nature it is difficult to isolate these well hydrated hydrophilic species from aqueous environments by a simple organic phase extraction.

A method for performing extractions of ionised solutes into organic phases has been studied for a number of decades, in which ions of opposite electrical charge are added to the aqueous phases resulting in ion-pairing between the solute ion and pairing ion. The resultant complex has a low net electrical charge or polarity, is thus poorly hydrated, and so now can transfer readily to organic phases. Jonkman[1,2] has reviewed the recent increased interest in bulk-phase extraction of ionised drugs by giving some 87 examples of solute extraction in which a selectivity and sensitivity of approach could be demonstrated. Perhaps the most sophisticated studies using the technique have been performed by Higuchi *et al.*[3,4] in Kansas, U.S.A., and by Schill and co-workers[5–7] at Uppsala, Sweden. Although preceded by attempts to use the phenomena for paper and thin-layer methods, over the past eight years many studies have been made to extrapolate ion-pair techniques into the HPLC mode, and only since small particle stationary supports were introduced have such approaches been of significance.

HPLC has been used previously for ion analysis using straight phase adsorption methods. This, however, often results in high solute retention coupled with very poor peak shape and solute resolution. (Poor peak symmetry is also caused by an alteration in the ionised/unionised form concentration ratio during separation, which necessitates the use of high buffer salt concentrations thus leading to column instability.) High-pressure ion-exchange chromatography has also been used for ion analysis, but the generally unfavourably high compressibilities of the materials, *e.g.* a polystyrene–divinylbenzene matrix cation exchanger, does not permit efficient high-speed separations to be made. The requirements of a HPLC method for the analysis of ionised solutes are rapidity, sensitivity, selectivity, efficiency and an ability to resolve material from complex systems, such as biological fluids, without a prior solvent extraction step.

II. ION PAIRS

The terms "ion pair" and "ion-pair formation" are often ill-used in connection with chromatographic systems. We define ion pairs (or triplets, etc.) as Coulombic association species formed between two ions of opposite electrical charge. Their formation, however, is dependent upon many variables including ion constitution and polarisability and solvent dielectric. For chromatographic purposes ion pairs will be formed between inorganic–organic and organic–organic solute pairs, and the formation of each type will be highly dependent upon the immediate environment of both ions.

The failure of sodium chloride to behave as a strong electrolyte in liquid ammonia first led Bjerrum[8] to postulate a theory of ion association based on electrostatic considerations. Such a theory took into account the fact that in solvents of low dielectric constant, ionophores (*i.e.* substances composed of ions which do not combine into covalently bonded molecules) of opposite electrical charge could as-

sociate whilst still retaining their basic properties, and that the association was caused by Coulombic forces and to a lesser extent by other interactions. This association of electrostatic charges leads to a net partial electrical neutrality, although it is important to understand that this is not complete and that ion-pairs still have some polarity[4]. Bjerrum's orginal model was proposed to account for the altered properties of strong electrolytes in solvents of low dielectric constant. Indeed it was believed originally that ion pairs could only exist in such solvents. However, since the studies of Diamond[9] it is now realised that in aqueous solution if both the cation and the anion are large, which generally means having some hydrophobic integrity, then there is a tendency for these large ions to be forced into a single, larger cavity by the water molecules so as to decrease the disturbance of its (the solvent's) structure. Thus ion-pairing in aqueous systems or in other highly structured (bonded) solvents is primarily enforced not by an electrostatic ion–ion interaction, as envisaged by Bjerrum, but is the result of solvophobic effects which then permit ion–ion association to occur.

The major property of ion pairs in an analytical context is their resultant ability to move from an aqueous environment to areas of lower dielectric. With ion-pair association the overall transfer between such phases will be determined by the extent of ion-pair formation, the nature of the formed species and the properties of the extracting phase. Thus $Na^{\delta+},Cl^{\delta-}$ will have a much lower oil solubility than say $R\cdot SO_4^{\delta-},R\cdot N^{\delta+}$.

Scheme 1 is a consideration of the overall phase transfer of ion pairs:

$$\underset{\text{aqueous}}{A^+ + B^-} \;\underset{|}{\overset{|}{\rightleftharpoons}}\; \underset{\text{organic}}{(A,B)}$$

Scheme 1.

For hydrophobic ions the formation of the ion pair will be in the aqueous bulk phase, followed by subsequent transfer, *i.e.*

$$\underset{\text{aqueous}}{R\cdot A^+ + R\cdot B^-} \overset{\text{fast}}{\rightleftharpoons} (R\cdot A,R\cdot B) \overset{\text{slow}}{\rightleftharpoons} \underset{\text{organic}}{(R\cdot A,R\cdot B)}$$

Scheme 2.

But for inorganic molecules and organic molecules of lower molecular weight, ion-pair formation will occur in the interfacial or diffusion layers between the two phases where the dielectric constant will be far lower than that of the aqueous phase. This is particularly important to note with regard to chromatographic systems, and is a consideration which is absent from most publications in the chromatographic literature. Since ion pairs are not non-polar, it is most relevant to discuss extraction of the species in terms of solvate formation rather than in relation to solvent dielectric constant or regular solution theory. For example, Higuchi *et al.*[4] have examined the extraction of dextromethorphan–hydrobromide pairs into various polar organic solvent-cyclohexane mixtures. For a chloroform–cyclohexane system the extraction ability far exceeds those of either a cyclohexanone–cyclohexane or nitrobenzene–cyclohexane system, although the dielectric constants for the chloroform–cyclohexane phases are lower. Regular solution concepts would tend to predict a greater extraction of the slightly polar ion pair into the more polar extracting phase.

The reason for this not being so is that the dextromethorphan–hydrobromide ion pair can be regarded as having a large, mostly lipophilic cation with a low net positive charge per unit area and a somewhat smaller anion which is expected to carry a relatively high net negative charge per unit area. This type of species would be expected to be solvated in the organic phase and extracted into it by molecules having an exposed positively charged surface, *e.g.* dipolar molecules with acidic protons such as chloroform, phenols and alcohols. In addition strongly hydrogen-bonding solvents such as lipophilic alcohols will have a high extracting ability for ion pairs having hydrophilic substituents, although we shall see later that such agents are far less selective in chromatographic systems than solvents having a much lower solvating ability[10,11].

For the distribution of a number of inorganic anion–organic quaternary cation pairs it has been demonstrated[12,13] that for a majority of anions studied, the distribution ratio generally increases with the size of the cation, and for a phosphonium cation series the value of the distribution ratio increases by a factor of about two for each additional methylene group in the alkyl chain. For these systems it has also been found that the order of extraction of the anion is:

$$ClO_4^- > SCN^- > I^- > ClO_3^- \geq NO_3^- > Br^- > BrO_3^- > Cl^-$$

Unlike transfer of uncharged solute molecules, the free-energy of transfer of ion pairs involving inorganic anions appears to be mainly enthalpically controlled, with a structuring within the organic phase being largely responsible for the net negative entropy measured.

Other factors altering the distribution of the ion pair include side reactions, polarisability and environmental factors such as temperature, ionic strength and pH.

III. ION PAIRS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

For the purpose of this paper the subject has been treated on the basis of the chemical nature of the pairing ion, *viz.* inorganic, organic and surface-active. Semantics are sometimes confusing. In this contribution we will identify the ion complexing with the sample as the *pairing ion*, which is analogous to the "heteron" term of Horváth *et al.*[14] and the pairing ion's "gegenion" will be called the *counter ion*. We also define a *partition process* as one which occurs between bulk phases and in which the influence of the boundaries of the phases has diminished to a negligible effect. Such a process is difficult to perceive in, at least, reversed-phase HPLC systems and solute transfer as ion pairs will be referred to as a *distribution* process.

III.1. Theoretical considerations

The distribution of an ion between two phases may be typified by examining the transfer of a sample or solute anion B^- as an ion pair A,B with a pairing cation A^+. We may write:

$$A_{aq}^{m+} + B_{aq}^{n-} \rightleftharpoons (A_n,B_m)_{org} \quad (1)$$

where m and n refer to the stoichiometry of the process, and the subscripts aq and org refer to the aqueous and organic phases. The extraction constant, $E_{A,B}$, for this

process may be written as:

$$E_{A,B} = [A_n,B_m]_{org} \cdot [A^{n+}]_{aq}^{-1} \cdot [B^{m-}]_{aq}^{-1} \quad (2)$$

Eqns. 1 and 2 assume that no significant ion-pair formation occurs in the aqueous phase, and to account for such water structure enforced pairing[9] $E_{A,B}$ would need to be modified. To handle side-reactions and competing or secondary equilibria which may occur, a distribution ratio, D, for the anion as the ion pair needs to be introduced. Since the distribution ratio is a stoichometric ratio of solute concentration in both phases, then assuming monovalent ions we have:

$$D_{A,B} = [A,B]_{org} \cdot [B^-]_{aq}^{-1} \quad (3)$$

or

$$D_{A,B} = E_{A,B} \cdot [A^+]_{aq} \quad (4)$$

The influence of side-reactions, etc. on the distribution ratio $D_{A,B}$ may be accounted for[5,7] by the use of α-coefficients according to Ringbom[15], such that a conditional extraction constant $E^*_{A,B}$ can be obtained from the total concentrations of each of the species, *i.e.*

$$E^*_{A,B} = C_{A,B_{org}} \cdot C_{A^+_{aq}}^{-1} \cdot C_{B^-_{aq}}^{-1} \quad (5)$$

where $C_{A,B_{org}}$ is the total concentration of B^+ extracted to the organic phase as ion pair with A^+, and $C_{A^+_{aq}}$ and $C_{B^-_{aq}}$ are the total concentrations of A^+ and B^- in the aqueous phases.

The use of a conditional extraction constant means that several simultaneous distribution processes can be treated simultaneously[16]. The conditional extraction constant can be related to the stoichometric extraction constant using the α-coefficients, *i.e.*:

$$E^*_{A,B} = E_{A,B} \cdot \alpha_{A,B} \cdot \alpha_{A^+}^{-1} \cdot \alpha_{B^-}^{-1} \quad (6)$$

where the values of α increase from unity as side-reactions, such as dimerization and dissociation of the ion pair in the organic phase, begin to occur. The distribution ratio now becomes:

$$D_{A,B} = E^*_{A,B} \cdot C_{A^+_{aq}} \quad (7)$$

To relate these parameters to conventional chromatographic terms the chromatographic capacity ratio, κ_i, of a component ion i needs to be introduced. κ is determined by the distribution cofficient, K_i, and the volume ratio, V_s/V_m, of the stationary and the mobile phase, *i.e.*

$$\kappa_i = K_i \cdot (V_s/V_m) = \frac{t_{Ri} - t_{R0}}{t_{R0}} \quad (8)$$

where V_m is the interstitial volume, V_s the volume of the stationary phase and t_{R0} the retention time of a non-retained compound. Thus by analogy between K and D, we may obtain:

$$\kappa_B = V_s \cdot V_m^{-1} \cdot E^*_{A,B} \cdot C_{A^+_{aq}} \quad (9)$$

where κ_B is the capacity ratio of the anion B^- chromatographed with the pairing ion A^+.

Eqns. 3–9 hold for reversed-phase HPLC systems when the sample retention is by ion-pair transfer from the mobile aqueous phase to the stationary organic phase.

Different equations can similarly be written for straight-phase ion-pair HPLC systems where the stationary phase is the aqueous phase. First $D_{A,B}$ needs to be defined in a reciprocal fashion to $E_{A,B}$, *i.e.*

$$D_{A,B} = [B^+]_{aq} \cdot [A,B]_{org}^{-1} \tag{10}$$

or

$$D_{A,B} = (E_{A,B} \cdot [A^+]_{aq})^{-1} \tag{11}$$

Using conditional extraction constants, then:

$$D_{A,B} = (E^*_{A,B} \cdot C_{A^+_{aq}})^{-1} \tag{12}$$

The capacity ratio for B^-, as the ion pair, is now given by:

$$\kappa_B = V_s \cdot (V_m \cdot E^*_{A,B} \cdot C_{A^+_{aq}})^{-1} \tag{13}$$

III.2. Significance of the extraction constant

As seen from eqns. 3–13 it is possible to write relationships between solute ion retention and solute ion extraction constants for both straight- and reversed-phase systems. In both cases the pairing ion is present initially in the aqueous phase. The question arises as to how the magnitude of the extraction constant relates to actual found capacity ratios. Figs. 1 and 2 show how the retention of an anion B^- is related to its capacity ratio when the anion is extracted as ion pairs of differing extraction ability (achieved by alteration in pairing-ion type, extracting phase solubilizing properties, etc.).

From eqns. 9 and 13 and Figs. 1 and 2 it may be appreciated that to obtain appropriate capacity ratio values (*i.e.* 1–50), and at pairing-ion concentrations between 0.01 and 0.1 mol·dm^{-3}, assuming a $V_s \cdot V_m^{-1}$ value of 1, then for straight-phase systems $E_{A,B}$ values of approximately 0.2–100 and for reversed-phase system $E_{A,B}$ values of 10–5000 are necessary. In practice, in reversed-phase systems at least, $V_s \cdot V_m^{-1}$ values are lower than 1.0 with the result that the idealised situation given by Fig. 2 needs to be modified towards higher $E_{A,B}$ values. Table 1 shows how $E_{A,B}$ may be regulated by judicious choice of either pairing ion or extracting phase. Although these values have been measured in bulk phase experiments and are from various sources, they illustrate the flexibility of the approach.

III.3. Column load capacity

With the addition of finite amounts of pairing ion to an HPLC system the question of column load capacity needs to be considered. It will be seen later (section IV.3.b) that this is most important when low concentrations of pairing ion are used. In most ion-pair HPLC systems (section IV.2.b) the load capacity factor is of importance when considering preparative-scale separations and the analysis of trace amounts. Eksborg and Schill[19] have described the theoretical relationships between the distribution ratio and buffer capacity in an ion-pair chromatographic system.

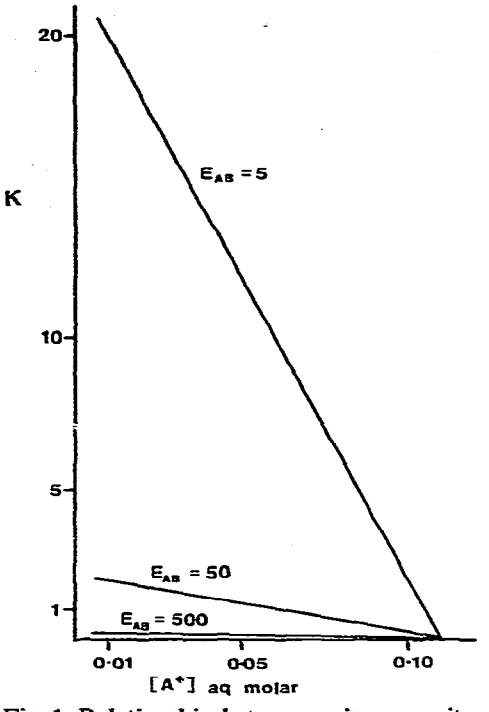

Fig. 1. Relationship between anion capacity ratio and pairing-ion concentration $[A^+]$ for different extraction constants and in a straight-phase HPLC system. $V_s \cdot V_m^{-1} = 1$.

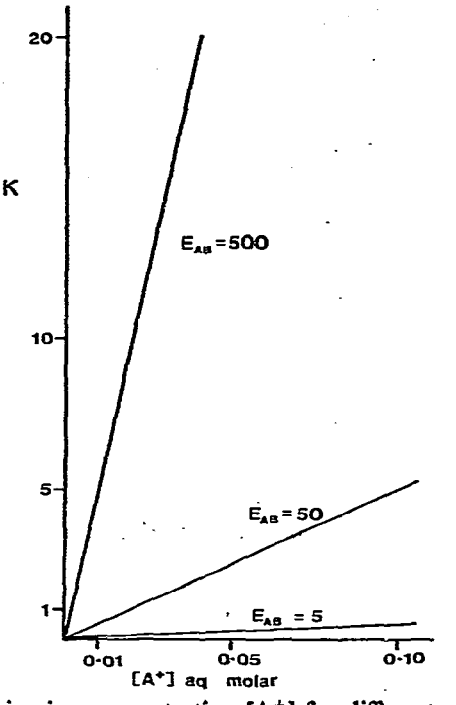

Fig. 2. Relationship between anion capacity ratio and pairing-ion concentration $[A^+]$ for different extraction constants and in a reversed-phase system. $V_s \cdot V_m^{-1} = 1$.

TABLE I

EXTRACTION CONSTANTS OF TETRAALKYLAMMONIUM–PICRATE ION PAIRS

TMA = tetramethylammonium; TEA = tetraethylammonium; TBA = tetrabutylammonium.

Cation	*Extracting phase*	*Log extraction constant*	*Reference*
TEA	Methylene chloride	2.34	17
TBA		6.68	17
TEA	Chloroform	1.32	18
TBA		5.91	18
TMA	Chloroform–1-pentanol	−0.51	19
TEA	(19:1)	1.46	19
TBA		6.60	19
TBA	Benzene	3.59	18
TBA	Carbon tetrachloride	5.91	18

The buffer capacity, β, of such an ion-pair system containing an anion sample B^- and the pairing ion A^+ is defined[19] by:

$$\beta = \frac{dC_{B_{org}^-}}{dp\,[A^+]} \tag{14}$$

where $C_{B_{org}^-}$ is the total concentration of B^-, and which is taken to be completely in the extracting phase.

In its simplest form, if A^+X^- is the pairing ion and its counter ion then it

follows that

$$C_{B_{org}^-} = [B^-]_{aq} \cdot q + [A,B]_{org} \tag{15}$$

and

$$[B^-]_{aq} = [A^+]_{aq} - [X^-]_{aq} \tag{16}$$

where q is the phase ratio term $V_s \cdot V_m^{-1}$. Thus it follows that

$$\frac{d([B^-]_{aq} \cdot q)}{d[A^+]_{aq}} = q \tag{17}$$

By combination of eqns. 15–17 with eqn. 2 Eksborg and Schill have been able to relate β to $E_{A,B}$ (see ref. 19 for full derivation). Thus:

$$\frac{\beta}{2.3} = -([A^+]_{aq} \cdot q + E_{A,B} \cdot [A^+]_{aq}^2 + [A, B]_{org}) \tag{18}$$

and from eqns. 11 and 13 for a straight-phase system it may be found that:

$$D_{A\,B} = \frac{K_B}{q} \tag{19}$$

It should be noted here that eqn. 19 is different from that first derived by Eksborg and Schill[19] in which $D_{A,B}$ is made equal to $q \cdot \kappa^{-1}$. This is because of the fashion in which, for straight-phase systems, we have defined $D_{A,B}$, *i.e.* in a reciprocal relationship to $E_{A,B}$ (eqn. 10). We believe this to be a preferable convention. Eqn. 19 now also holds for reversed-phase systems (eqns. 4 and 9). Table 2 uses eqns. 10, 15, 18 and 19 to show how buffer capacities of a straight-phase ion-pair HPLC system are regulated by different extraction constants. From this it is seen that the higher is the extraction constant then the lower is the system's load capacity, so leading to an unstable system with non-constant properties. Conversely a low extraction constant gives rise to a higher load capacity. Eksborg and Schill[19] have pointed out that the negative sign of the buffer capacity, as defined, illustrates that an increase in sample concentration will decrease pA^+. Thus for the production of a stable system when the prime mechanism of retention is ion-pair distribution a relatively high concentration of the pairing ion is necessary.

TABLE 2

RELATIONSHIP BETWEEN BUFFER CAPACITY OF A STRAIGHT-PHASE ION-PAIR SYSTEM AND EXTRACTION CONSTANTS

Sample concentration $1 \cdot 10^{-6}$ mol·dm^{-3}, $q = 1$, $\kappa = 10$.

$E_{A,B}$	$[A, B]^*_{org}$	$[A^+]^{**}_{aq}$	β^{***}
$1 \cdot 10^3$	$9.09 \cdot 10^{-8}$	$1 \cdot 10^{-4}$	$-2.42 \cdot 10^{-4}$
10	$9.09 \cdot 10^{-8}$	$1 \cdot 10^{-2}$	$-2.53 \cdot 10^{-2}$

* Obtained by eqns. 15 and 19.
** Obtained by eqns. 10 and 19.
*** Obtained by eqn. 18.

IV. PAIRING IONS

Eqns. 7, 9, 11 and 13 show that the distribution ratio of an ion as an ion pair is dependent upon its extraction constant as an ion pair, the concentration of the pairing ion, and the phase volume ratio. In this section the relationship between these three parameters is more closely considered especially with regard to the nature of the pairing ion, the properties of the extracting system and the environmental factors such as pH, temperature and ionic strength which can affect the chromatography. It is possible to group the pairing ions into three main types, inorganic, hydrophobic and surface-active.

IV.1. Hydrophobic ions

It is convenient to describe the use of hydrophobic ions in HPLC at this stage, although it will be realised from section III.2 that historically the development of their use came primarily after that for inorganic ions.

Generally this form of ion-pair HPLC uses an aqueous mobile phase and an inorganic solvent as stationary phase. The support for this stationary "phase", however, may be either of a conventional silica type composition or of a "reversed-phase" material such as octadecylsilane chemically bonded to silica. Reversed-phase "partition" chromatography, which was first developed by Howard and Martin[20], had, until the introduction of sophisticated support materials, been severely restricted owing to the inability to obtain adherence of an organic "phase" onto the support.

Coating of the stationary phase has been performed by a number of methods. For example the stationary phase has been applied onto siliconized cellulose by mixing the support and the stationary liquid before the packing of the column, although *in situ* methods are more commonly used in which the stationary liquid phase is applied to the packed column by adsorption from a mobile phase.

Table 3 lists those ions which have been used as hydrophobic pairing ions in HPLC. The polarity of the sample solute is the initial consideration when determining an appropriate pairing ion. It can be seen from Table 3 that around seventeen ions may be classed as being hydrophobic and small (although this classification is not strict), and half of these are appropriate for the resolution of anionic solutes.

IV.1.a. Small alkylammonium ions

The interest in the use of small hydrophobic alkylammonium ions undoubtedly results from the pioneering studies of Wittmer *et al.*[21] and of Schill and others[6,7]. Wittmer *et al.* have extensively studied systems using tetrabutylammonium ion as the pairing agent. (This, along with other ions is marketed by Waters Assoc.[22] as a pairing-ion reagent.) Alkylammonium ions have the property of being aprotic and may therefore be used at all pH values; since they are both hydrophobic and ionised, they will be able to form water structure enforced ion pairs[9] in environments having a high dielectric constant, although this tendency will be reduced the shorter the alkyl chain is. The hydrophobic character of these ions is easily altered by changing the length of the alkyl chain, and thus a manipulation of the capacity ratio will be achieved by the alteration in the extraction constant. Addition of a methylene unit to a solute raises its oil–water partition coefficient by about 0.5 log units. However, the addition of an increasing number of methylene units to the pairing ion will reduce the total solubility of the formed ion pair. The solubility of the pairing ion is an important consideration and not only for operational purposes (*i.e.* column blockage etc.). For example, in reversed-phase systems although acetonitrile is a

TABLE 3

PAIRING IONS, SOLUTES AND CHROMATOGRAPHIC SYSTEMS STUDIED

Phase system				*Solute or solute class*	*Reference*
*Pairing ion**		*Support***	*Eluent and stationary phases****		
(i) Small alkylammonium ions					
Tetramethylammonium	(E)	Micropak RP-18 (10 μm)	Methanol–water	Alkylbenzenesulphonates Sulphonated dyes	23
	(E)	Silica RP-18 (10 μm)	Methanol–water	Hydrocortisone and salts	80
Tetraethylammonium	(S)	LiChrosorb/cellulose	Butanol–methylene chloride/ Tris, borate or carbonate buffers	Phenylacetic acids	65
	(S)	DiaChrom (37–44 μm)	*n*-Hexane–isobutanol	Tricyclic antidepressants	67
	(E)	μBondapak C_{18}	Methanol–water	Hydrocortisone and salts Tartrazine and salts	80 21
Tetrapropylammonium	(E)	μBondapak C_{18}	Methanol–water	Hydrocortisone and salts	80
	(E)	LiChrosorb RP-2, RP-18 (10 μm)	Pentanol/phosphate buffer (pH 7.5)	Steroid glucuronides	95
	(E)	LiChrosorb RP-2, RP-8, RP-18 (10 μm)	Pentanol/phosphate buffer (pH 7.4)	Carboxylic and naphthylacetic acids	96
Tetrabutylammonium	(S)	Silica (6 μm)	Butanol–*n*-hexane-methylene chloride	Carboxylic acids	25
	(S)	Silica (6 μm)	Butanol–*n*-hexane/borate buffer (pH 9.2)	Sulphonamides	32, 33, 81
	(S)	Silica	Dichloromethane–1-pentanol	Glucuronides, oestrogens	26
	(S)	LiChrosorb SI 100 (10 μm)	Butanol–methylene chloride–*n*-hexane/Tris buffer (pH 8.3)	Indoleacetic acids	50
	(S)	LiChrosorb/cellulose	Butanol–methylene chloride/ Tris, borate or carbonate buffer	Phenylacetic acids	65
	(E)	μBondapak C_{18}	Methanol–water (pH 5.0)	Ascorbic acid	72
				Tartrazine and salts	21
				Hydrocortisone and salts	80
				Folic acid derivatives	97
				Thyroidal iodoamino acids	98
				N-Substituted pteroylglutamic acids	99

	(E)	LiChrosorb RP-2 (10 μm)	Butyronitrile/phosphate buffer (pH 7.4)	Sulphonamides, barbituric acids	100
	(E)	Corasil C_{18} (37–50 μm)	Methanol–water	Thyroidal iodoamino acids	98
	(E)	ODS/TMS (7 μm)	Water–methanol–formic acid	Paracetamol and its metabolites	101
	(E)	Silica RP-18	Methanol–water	Carbazoles	82
				Nitrophenols	83
	(E)	Silica RP-18	Pentanol–butyronitrile/ phosphate buffer (pH 7.4)	Sulphonamides	26, 81
				Barbituric acids	81
	(E)	Silica RP-18/ Silanised silica SI 60	Pentanol/phosphate buffer (pH 7.4)	Benzoic and sulphonic acids	26, 27
				Nicotinic acid	27
	(E)	Silica RP-2	Pentanol/phosphate buffer (pH 7.4)	Mandelic, nicotinic and acetic acids	66
Tetrapentylammonium	(S)	Silica	Methylene chloride–*n*-hexane–butanol/buffer (pH 7.4)	Phenylacetic acid	65
	(S)	Silica	Dichloromethane-pentanol/ buffer (pH 7.4)	Glucuronides, oestrogens	26
	(E)	μBondapak C_{18}	Methanol–water	Hydrocortisone and salts	80
(ii) Picrate	(S)	Cellulose	Dichloromethane-pentanol/ water	Acetylcholine, choline	41, 42, 53, 68
	(S)	Kieselguhr/ Merckosorb SI 100 (5 μm)	Chloroform saturated with picric acid	Belladonna alkaloids	39, 40
				Tropa alkaloids	102
	(S)	Ceria microspheres (5–40 μm)	Pentanol–chloroform	Alkylammonium ions	103
	(S)	Cellulose	Chloroform–aliphatic alcohols/ water	Quaternary ammonium salts	53, 19
(iii) Alkylsulphonates, etc.					
Methanesulphonate	(S)	Silica (10 μm)	*n*-Hexane–methylene chloride–butanol/water	Tricyclic antidepressants	49, 50
Pentanesulphonate	(E)	Chromomegabond RP-8	Methanol–water–glacial acetic acid	Diminazene derivatives	48
Hexanesulphonate	(E)	μBondapak C_{18}	Methanol–water	Adrenaline	104
	(E)	Silica RP-18	Methanol–water	Peptides	105

(Continued on p. 326)

TABLE 3 *(continued)*

Phase system				*Solute or solute class*	*Reference*
*Pairing ion**		*Support***	*Eluent and stationary phases****		
Heptanesulphonate	(E)	μBondapak C_{18}	Methanol–water	Niacin, niacinamide	72
				Opium alkaloids	84
				Glutathione, cysteine, etc.	85
				DL-Noradrenaline and metabolites	106
				Phenformin	107
				L-DOPA metabolites	108
	(E)	μBondapak C_{18}	Acetonitrile/aqueous buffer	Diazonium salts	47
				Atropine sulphate and other anticholinergics	43–46
	(E)	μBondapak C_{18}	0.1 *M* Na_2HPO_4 (pH 5.0) and 0.1 m*M* EDTA	Catecholamines	109
	(E)	μBondapak CN	Acetonitrile–water–acetic acid	Phenylpropanolamine, bromopheniramine and other antihistamines	110
Toluenesulphonate	(E)	Silica RP-18 (10 μm)	Methanol–water	Quinidine	111
Camphorsulphonate	(E)	μBondapak C_{18}	Methanol–water (pH 2.5)	Niacin, niacinamide	72
Dioctylsulphosuccinate	(E)	μBondapak C_{18}	Methanol–water (pH 2.5)	Isoniazid, pyridoxine HCl	86
				Niacin, niacinamide	72
Cyclohexylsulphamates (+ long-chain alkylammonium ions)	(E)	LiChrosorb RP-8, RP-18 (10 μm), RP-8 (5 μm), Spherisorb ODS (5 μm)	Pentanol/aqueous buffers	Organic ammonium ions (hydrophobic amines)	112, 113
(iv) Specific ions					
Naphthalene-2-sulphonate	(S)	Cellulose/Celite Diachrom	Chloroform–pentanol/phosphate buffer (pH 2.4)	Primary and secondary amines	54
	(S)	LiChrospher SI 100 (10 μm)	Chloroform–pentanol/phosphate buffer (pH 2.4)	Amino acids, peptides, alkylamines	56
N-Methylimipramine	(S)	Cellulose (35–70 μm)	Hexane–methylene chloride–pentanol/carbonate or phosphate buffer (pH 9)	Benzilic, salicylic and phenylbutanoic acids	52
N,N-Dimethylprotriptyline	(S)	Cellulose (35–70 μm)	Cellulose (35–70 μm)	Benzilic, salicylic and phenylbutanoic acids	51, 53
	(S)	LiChrospher SI 100 (10 μm)	Chloroform–pentanol/water	Alkylsulphates, alkylsulphonates	56

(v) Inorganic ions					
Perchlorate	(S)	LiChrosorb SI 100 (10 μm)	Butanol–methylene chloride–*n*-hexane/water	Quinidine and quaternary ammonium analogues	
	(S)	LiChrosorb SI 60 (7 μm), SI 100 (10 μm), Partisil 5	Methanol–dichloroethane/water	Disopyramide, lidocaine, tocainide	115
	(S)	Partisil 5	Methanol–dichloroethane–diisopropyl ether/water	Amitriptyline, nortriptyline + stereoisomers	116
	(S)	LiChrosorb SI 60 (5 μm)	Methanol–methylene chloride–hexane/water	Quinidine	117
				Metanephrine and normetanephrine	50, 114
	(S)	Silica (4 or 10 μm)	Butanol–methylene chloride/water	Thyroid hormones	33
	(S)	Silica (6 μm)	Tributyl phosphate–hexane/water	Catecholamines	25
	(S)	Merckosorb SI 100 (10 μm)	Chloroform–aliphatic alcohols/water	Phenothiazines, dibenzazepines	59
	(S)	Spherisorb S5	Halogenated hydrocarbons–aliphatic alcohols/water	Catecholamines	61
	(S)	Cellulose	Cyclohexane–pentanol/water	Amino acids, biogenic amines	10
				Alprenolol and metabolites	35
	(E)	ODS/TAS (RP)	Acetonitrile–aqueous citric acid	Tetracyclines	87
Perchlorate (E) + tri-*n*-octylamine (S)		Silica	Tri-*n*-octylamine-water	Sulphonic and carboxylic acids, phenols	62
				Salicylic acid + other anti-inflammatory drugs	118
Chloride	(S)	Cellulose	Pentanol–cyclohexane/water	Amino acids	10
	(S)	DiaChrom (37–44 μm)	Pentanol–chloroform/water	Alkylammonium salts	55
	(S)	Partisil 10	Butanol–hexane/water	Phenothiazines	119, 120
	(E)	Cellulose	Pentanol/water	Emepronium and derivatives	63
Bromide	(E)	Cellulose	Pentanol/water	Emepronium and derivatives	63
	(E)	Silica RP-8, RP-18 (10 μm) Spherisorb ODS (5 μm)	Pentanol/aqueous buffers	Organic ammonium ions	112, 113
Phosphoric acid/phosphate	(E)	μBondapak C_{18} (10 μm)	Methanol–water	Thyroidal iodoamino acids	98
		μBondapak-fatty acid		Peptides	105, 121
	(E)	RP Silicas	Pentanol/aqueous buffers	Hydrophobic amines	112, 113
Nitrate or formate	(E)	ODS-silica	Methanol–water	Erythromycin, diamines, benzocaine	122

TABLE 3 *(continued)*

Phase system				*Solute or solute class*	*Reference*
*Pairing ion**		*Support***	*Eluent and stationary phases****		
(vi) Surface-active ions					
Tridecylammonium	(E)	μBondapak C_{18}	Methanol–water (pH 7.5)	Hydrocortisone and salts	80
Cetyltrimethylammonium	(E, S)	Spherisorb ODS (5 μm)	Propanol–water	Labetalol	75
		SAS silica (RP) (7 μm)	Propanol–water	Sulphonic acids and dyestuffs	70
	(E)	Silica	Propanol–methylene chloride–water	Sulphonic acids and dyestuffs	70
		Ceria microspheres (5–40 μm)	Methanol–water	Sulphonic acids	103
Dioctylammomium	(S)	ODS/TMS silica (RP)	Water–methanol–formic acid	Paracetamol and conjugated metabolites	101
Alkylbenzyldimethylammonium chlorides	(E, S)	Spherisorb ODS (5 μm)	Methanol–water	Bischromones, acid red dyes, benzoic acids	74, 78
Octylsulphate	(E)	RP silicas	Pentanol/aqueous buffers	Organic ammonium ions	113
Decylsulphate	(E)	LiChrosorb-RP-18 (5 μm)	Phosphate buffer (pH 2.1)	Hydrophilic amino acids	76, 77
Dodecylsulphate	(E, S)	RP-2, RP-8, RP-18 methyl silicas	Citrate buffer–*n*-propanol (*e.g.*)	Amino acids	
Dodecylsulphonate	(E, S)				
Dinonylnaphthalenesulphonate	(E, S)				
Dodecylsulphate	(E, S)	Micropak RP-18 (10 μm)	Acetonitrile–water	Catecholamines	73
Dodecylsulphonate	(E, S)				23
Dodecylsulphate	(E, S)				
1-Dodecanesulphonate	(E, S)	ODS/TMS silica (RP)	Sulphuric acid–water–methanol (or acetonitrile)	Catecholamines and metabolites	
Dodecylbenzenesulphonate	(E, S)				61
Decylsulphate	(E, S)				
Dodecylsulphate	(E, S)	Spherisorb ODS (5 μm)	Methanol–water	Triazines	88
Tetradecylsulphate	(E, S)			Tryptophan and metabolites	123
(vii) Trichloroacetate	(E)	μBondapak C_{18}	Acetonitrile–water	β-Endorphins	
	(E)	Alkylphenyl-μBondapak	Methanol–water	Benzomorphans, methadone hydrochloride	124

* E and S refer to the nominal position of the pairing ion in either the eluent or stationary phase as indicated by the respective authors.

** RP refers to chemically bonded reversed-phase stationary supports.

*** Eluent and stationary phases indicated are typical examples.

preferred solvent owing to its low viscosity and specific solvating properties, its solubilizing properties for many of the common alkylammonium ions often limits its use. As a result methanol–water eluents are often employed as a compromise solvent. Gloor and Johnson[23] have pointed out that the tetraalkylammonium salts which are often used in the chloride salt form, can be used with acetonitrile in the eluent if higher water contents are used; however, this places an upper limit on the range of organic modifier which can then be used in gradient elution. It would be preferable of course to use the more soluble bromide or iodide salt forms, although their high absorbance at low wavelengths may limit their use. One problem with using alkylammonium ions is the buffer salts which are necessary to obtain the desired pH, for it should be appreciated that these too can ion-pair with the ionised solute. In addition, it may be shown[24] that an increase in ionic strength decreases the transfer of ion pairs between water and organic solvents *i.e.* a "salting-in" effect rather than the more normal "salting-out" phenomenon found with neutral molecule or weakly polar molecule partitioning.

It has already been shown (see section III.1) that $E_{A,B}$ values will help determine the chromatographic behaviour of ions in an ion-pair system. To maintain the capacity ratios at an acceptable level it is possible to choose an organic solvent whose solvation of the formed ion pair is at an appropriate level. Using tetraalkylammonium ions, conditions are usually selected for ion-pair distribution between the eluent and stationary phase to dominate, so that other forms of the solute in the organic phase become insignificant. For example, in the separation of carboxylic acids[25] using alkylammonium ions, the lower pH limit depends upon the point at which the undissociated acid distributes significantly into the organic phase, whilst the upper limit is generally set by the possible side-reactions taking place. With phenolic carboxylic acids an upper limit of between pH 7 and 10 is considered reasonable, with the controlling influence the protolysis of the phenolic group.

The choice of either a straight silica-type or reversed octadecylsilane-type stationary support material can be a confusing one. Fransson *et al.*[26] have made a comparison of reversed- and straight-phase ion-pair HPLC systems and conclude that both give good possibilities for the separation of acidic organic compounds using tetraalkylammonium ions as pairing ions. Significantly they point out that reversed-phase techniques have the undoubted advantage of a better control of retention by change in the type and concentration of the counter ion in the mobile phase. Wahlund[27] has shown also that with reversed-phase systems the capacity ratios of anions using a tetraalkylammonium pairing ion can be regulated (and hence separation can be improved), by use of both gradient elution techniques and by direct injection of large sample volumes. Such approaches with straight-phase systems lead to column instability. The study by Fransson *et al.*[26] has included an examination of two different reversed-phase supports, *i.e.* a LiChrosorb RP-2 and a RP-18 system. RP-2 is a partly hydrophobized porous silica microparticle, whilst RP-18 is a hydrophobic material. Fransson *et al.* tested two stationary phases, *i.e.* a hydrogen-donating and -accepting solvent (1-pentanol), and a hydrogen-accepting solvent (butyronitrile). Using benzoic and benzenesulphonic acids they have shown that separations using all the possible combinations of support and phase are good, so good in fact that even the positional isomers of hydroxy- and aminobenzoic acid could be well separated. Fig. 3 shows that using the weakly hydrogen-accepting solvent butyronitrile a slightly better separation of these acids is achieved, however the influence of the support material is greater. Fig. 3 clearly shows that the benzoates are more retarded

than the corresponding sulphonates when RP-2 is used, although this material produced very strong peak tailing for some dihydroxylated acids, an effect which was eliminated when RP-18 was used. It may be seen from Table 3 that generally a C_{18} reversed-phase support material is preferred. Interestingly, Fransson *et al.*[26] also studied reversed-phase systems with no organic modifier present. Not surprisingly, they found very poor separating efficiency with RP-18 though the RP-2 material gave good efficiencies.

The use of porous silica microparticles has been examined as a possible stationary phase support using an aqueous stationary phase. Fransson *et al.* have found that unless the stationary aqueous phase is extremely well coated, and/or the mobile phase used is not perfectly saturated with the stationary phase, then drastic column instability occurs when tetraalkylammonium pairing ions are used. Eqns. 1–13 assume that the support material is so inert that it has only a minor or negligible influence on the migration rate of the sample ion. Eksborg and Schill[28] have presented data to show the effect of two straight-phase supports of low adsorptive power, *i.e.* diatomaceous earth (Celite) and cellulose (Brana Munktell 410). Using tetraalkylammonium and alkylamine pairing ions as stationary phase, with chloroform–1-pentanol as mobile phase, and with the sample being injected into the systems as the ion pair, it is found that (a) peak symmetry is better using cellulose compared to diatomaceous earth, and (b) that the derived distribution ratios for the samples are also lower on cellulose, indicating a lower migration rate. This latter effect has been postulated as being due to the greater ability of cellulose to bind the components of the ion pair by hydrogen bonding.

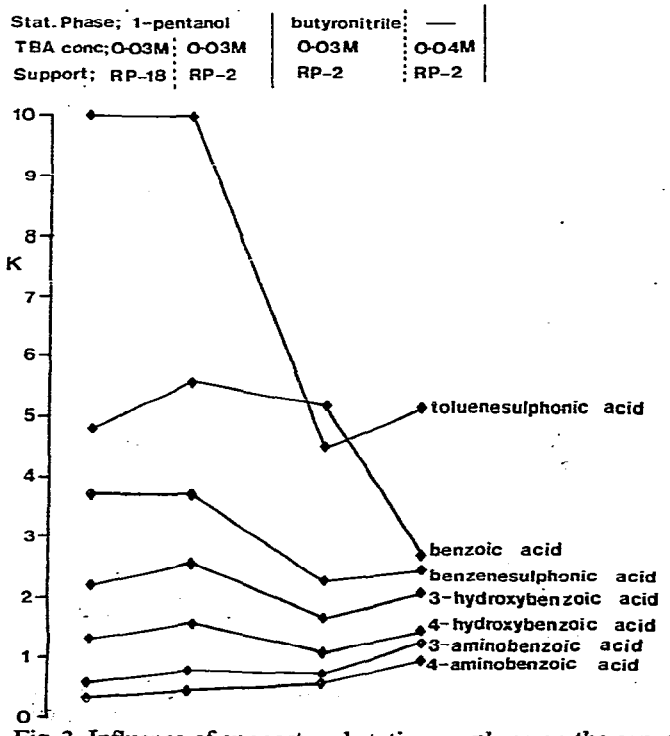

Fig. 3. Influence of support and stationary phase on the capacity ratios of benzoic and benzenesulphonic acids using reversed-phase ion-pair HPLC (from ref. 26). Mobile phase tetrabutylammonium ion (TBA) (pH 7.9).

Aliphatic alcohols generally constitute a major part of the phase system when tetraalkylammonium pairing ions are used. The Uppsala group prefers 1-pentanol as stationary phase with reversed systems, and 1-pentanol–chloroform mixtures as the mobile phase with straight-phase systems. Consideration of Figs. 1 and 2 and Table 1 will show why this is so with respect to both the extracting abilities of these solvents and the different range of $E_{A,B}$ values required for either straight- or reversed-phase procedures. One major problem with the use of polar solvents such as 1-pentanol in ion-pair HPLC is the commonly found side-reaction of dissociation of the ion pair in this organic phase[19]. This may be represented by eqn. 20.

$$A,B_{org} \rightleftharpoons A^+_{org} + B^-_{org} \tag{20}$$

Dissociation constants of around $1 \cdot 10^{-3}$ mol·dm^{-3} have been reported[11] for alkylammonium ion pairs in 1-pentanol. For such systems the conditional extraction constant (eqn. 5) may be written as[27]:

$$E^*_{A,B} = E_{A,B} \cdot \alpha_{A,B} = E_{A,B} \cdot (1 + K_{diss(A,B)} \cdot [A^+]^{-1}_{org}) \tag{21}$$

were $K_{diss(A,B)}$ is the ion-pair dissociation constant defined as:

$$K_{diss(A,B)} = [A^+]_{org} \cdot [B^-]_{org} \cdot ([A,B]_{org})^{-1} \tag{22}$$

If A,B is the only ion pair in the organic extracting phase, then both the concentration of pairing ion A^+ in this organic phase, and the capacity ratio will change with the concentration of sample B^-. This will lead to tailing peaks in the chromatogram, although if the concentration of pairing ion A^+ in the organic phase can be kept constant then the conditional extraction constant $E^*_{A,B}$ will be unaltered as sample concentration changes. We shall see in section IV.3.b how this may be achieved by loading the system with additional pairing-ion species.

A further problem with the use of aliphatic alcohols is their known self-association in both organic[29,30] and aqueous[31] solvents. Although this has no real effect on the chromatography of a compound, it does make elucidation of mechanisms and equilibrium difficult. For example, Su *et al.*[32] have shown that an increase in the 1-butanol mobile phase concentration in a tetrabutylammonium pairing-ion system with silica (LiChrospher SI-100) as stationary support at pH 8.5 causes a decrease in the capacity ratios of a series of sulphonamides. For all ionised sulphonamides studied the slope of the log–log plot between capacity ratio and 1-butanol concentration was found to be −3.4, which is in agreement with earlier work from this group[33] for studies at pH 9.2 using Merkosorb SI-100 as the stationary support. For the unionised drug sulfanilamide a slope of −2.55 was observed. Higuchi *et al.*[34] have considered the slope values for such plots to be the average solvation number of the ion pair in the organic phase. Thus eqn. 1 may be extended to take this solvation into account, *i.e.*

$$A^+_{aq} + B^-_{aq} \rightleftharpoons (A,B)_{org} \overset{+nROH_{org}}{\rightleftharpoons\!\!\rightleftharpoons} (A,B) \cdot (ROH)_n \tag{23}$$

where for 1-butanol R = 4. Thus sulfanilamide has a much lower solvation by the 1-butanol than the ionised sulphonamide. Eqn. 23 and the apparent solvation numbers found by Karger *et al.*[33] reveal three important effects. First, the solvation number is critical in effecting reproducible conditions. Thus if the phases are poorly prepared,

for example if mutual phase solubility has not been achieved, then the solvation number will alter and so will the capacity ratio. Second, environmental factors such as temperature and ionic strength, by their alteration of the solvation number, should be carefully controlled, and third, the high solvation numbers achieved with 1-butanol indicate that alteration in capacity ratios may be readily achieved by changing the organic phase composition.

Unfortunately little work has been reported on the effect of temperature and ionic strength in ion-pair HPLC systems. Su *et al.*[32] have briefly examined the effect of salt concentration on the chromatography of sulphonamides using a tetrabutylammonium pairing ion in a straight-phase system with 1-butanol–heptane as mobile phase. Although results were difficult to assess, they found that the capacity ratio decreased by a factor of two to three as the ionic strength was approximately doubled. With regard to selectivity they further point out that salt effects can be an influence depending on the ionization of the drug. We have found[24] that an increase in ionic strength decreases the transfer of cromoglycate–alkylbenzyldimethylammonium ion pairs from water into chloroform. Obviously when one considers the large number of additional equilibria which can occur in the more exotic HPLC systems, such a simple salting-in effect may not always be evident.

Eqns. 9 and 13 show that as the concentration of pairing ion changes the capacity ratio will alter also. It is expected *a priori* that a linear relation should exist between capacity ratios and pairing ion concentrations (Figs. 1 and 2), and for a large number of studies this has been the case. For example, with the previous sulphonamide example[32], double log plots of capacity ratio *versus* reciprocal of tetrabutylammonium ion concentration show that for completely ionised sulphonamides linearity is observed. This suggests that ion-pair formation and distribution are the sole retention mechanisms occurring. Similarly, Wahlund[27] has observed that the capacity ratio of the hydrophobic ion pair formed between toluenesulphonate and tetrabutylammonium rises from zero to 28 in a reversed-phase system with 1-pentanol as the stationary phase with a change in the pairing ion concentration from zero to 0.05 molar. This contrasts with a change in capacity ratio from zero to 3.6 for the hydrophilic ion pair formed between 4-hydroxybenzoate and tetrabutylammonium. Non-linear relationships do, however, exist[26] and may be attributed in part to the fact that side-reactions are not taken into consideration in eqns. 9 and 13. These lead to an alteration in the conditional extraction constant and can be due to both ion pairing in the aqueous phase and ion-pair dissociation in the organic phase. Thus an increase in the pairing ion concentration will decrease $E^*_{A,B}$ (eqn. 6). Ion-pair dissociation will dominate at low pairing ion concentration, and ion-pair formation in the aqueous phase will dominate at higher concentrations. Fransson *et al.*[26] have shown a non-linear relation between the capacity ratio of 4-hydroxybenzoic acid and the tetrabutylammonium ion mobile phase concentration in a reversed-phase 1-pentanol system. Non-linearity here is probably due to association equilibria in the aqueous phase between both the pairing ion and sample anion, and pairing ion and those buffer anions present in the system.

For a straight-phase system where the stationary aqueous phase of pairing ion has been pre-loaded onto the column, regulation of sample retention by changing the pairing ion loading is more difficult to achieve practically. Once a reasonable coating has been made, it is better to focus on the composition of the mobile phase to control retention although a problem with this approach is that a change in se-

lectivity can also take place[35,36]. It is common for straight-phase systems to use organic phases which consist of one weak and one strong solvating agent, with the result that interaction between the ion pair and the strongly solvating component can be treated[37,38] as a complexation process which may then be utilized as a retention regulator.

IV.1.b. Picrate ions

For cationic samples the use of picrate and its analogues as pairing ions has been favoured. Table 3 lists a number of significant studies where their use has been optimised. Owing to the electron-withdrawing effect of the nitro groups picric acid is a strong acid; because of this and its ability to form ion pairs with many solutes, including, for example, amines and quaternary ammonium ions, it has been used in straight-phase ion-pair HPLC. Schill and Eksborg[7,19] have shown that picrate ions loaded onto an ethanolysed cellulose support at pH 11.2 can separate well quaternary ammonium ions using chloroform–1-pentanol as mobile phase. Table 1 gives some $E_{A,B}$ values for picrate–alkylammonium ion pairs. Frei and co-workers[39,40] have applied the principles outlined by Schill's group in the use of picrate pairing-ions for the separation of some plant alkaloids using rigid stationary support particles. They demonstrate a specificity with their picrate system such that a separation of the alkaloids hyoscyamine, scopolamine and an ergot alkaloid can be observed 3–4 min after injection as the picrate ion pair. The stationary phase in this study consisted of pieric acid dissolved in a citrate buffer. Concentrations of picric acid used were 0.01 and 0.03 mol·dm^{-3}. As follows from eqn. 13 they found that an increase in picrate concentration decreased alkaloid capacity ratios; also that an increase in pH (which results in a lower concentration of the protonated alkaloid) led to higher capacity ratios. This is because a lower rate of ion-pair formation occurs causing a decrease in $E_{A,B}$ (eqn. 13-. Table 1 shows that if the polarity of the mobile phase were to be increased, $E_{A,B}$ would also increase, and hence the capacity ratio would fall. Although this is an attractive possibility, is does lead to detection difficulties caused by background changes. Frei and co-workers also chose picrate ions since they could use the high chromophoric property of this ion for the detection of the poorly chromophoric alkaloids. In contrast to earlier studies by Karger *et al.*[33] on the separation efficiencies of perchlorate and tetraalkylammonium pairing-ion systems using thyroid hormones and sulpha drugs as sample ions, Frei and co-workers had a prime aim of improving the detection limit for the plant alkaloids, and they estimate that about a 500-fold increase in alkaloid concentration would be required to produce the same signal if a non-chromophoric pairing ion had been employed. Classifying the technique as being complementary to true pre-column derivatization these workers point out that the technique is non-destructive and could be used for preparative purposes, for example, to carry out structure identifications.

These workers[39] also examined a wide range of possibly suitable stationary supports operated at low and high pressures and concluded that silica gel (pore size 1000 Å, particle size 5 μm) was best suited with regard to selectivity and stability for routine analysis. Interestingly they observed that with SI 1000 lower capacity ratio values resulted than with SI 100 (probably owing to the lower surface area of the former —15 m^2·g^{-1} compared to 300 m^2·g^{-1}).

The picrate pairing-ion system was first used[41] with cellulose microcolumns for the isolation of acetylcholine co-extracted with choline ester, etc. from brain homogenates. Picrate was used since it gave extraction constants of suitable size and could be used at a lower pH than the other anions which had been previously

examined with bulk extraction systems. As with all the early studies (Table 3) the emergence of assymetrical peaks at higher retention times was seen. These may be avoided by using chloroform as a specific solvating agent. Eksborg and co-workers[19,42] have further reported on the isolation of acetylcholine using picrate with chloroform–1-pentanol as mobile phase and 0.03 or 0.06 mol·dm^{-3} picrate as stationary phase; they were able to produce variations in capacity ratio by use of homologous alkylammonium picrates with differing $E^{*}_{A,B}$ values as samples. Changes in capacity ratio could also be achieved by varying the picrate concentration, but as discussed in the previous section this method is much less suitable since to obtain a high capacity ratio, for example, a very low picrate concentration would be required which results in an unstable system. In section III.3 it has been shown that for a stable chromatographic system which needs to have a high sample loading, this may be achieved if there is a high pairing-ion buffer capacity *i.e.* $\beta = dC_B^-/dp[A^+]$. For straight-phase systems this can only be achieved by a high pairing-ion concentration in the stationary phase (Table 2).

The high selectivity of picrate columns is illustrated by the fact that using chloroform–1-pentanol (19:1) as mobile phase, a separation factor of approximately 4 for each methylene group can be achieved, which is particularly useful in the isolation of similar molecules such as acetylcholine and choline.

With reference to eqn. 12 for straight-phase systems then using picrate (P^-) as pairing ion the total distribution of an amine in its protonated (AH^+) and free base (A) forms, may be given by:

$$D = (E^{*}_{P^-,AH} \cdot C_{P_S^-})^{-1} + D_A \qquad (24)$$

where D_A is the distribution of the free base. Usually the conditions are chosen so as to give negligible retention of the non-charged base. The problem encountered with picrate, and most stationary phase situated pairing ions, is that the high pH ranges often necessary to ensure negligible distribution of the free base cause bleeding of picrate from the column. Picrate columns also suffer from the disadvantage of picrate ion pairs having high dissociation constants (*ca.* $1 \cdot 10^{-4}$ mol·dm^{-3}), although it will be shown in section IV.3.b how this effect may be diminished.

IV.1.c. Alkylsulphonates

Alkylsulphonates have been used for the resolution of cationic species in a similar manner to the use of tetraalkylammonium ions for the resolution of anionic species. Undoubtedly the availability of a commercial "ion-pair reagent" product containing 1-heptanesulphonic acid has contributed to their usage. Generally aqueous acetonitrile mixtures[43–47] have been used as mobile phases using reversed-phase stationary supports. In all cases the mechanism of retention in such systems is described as "partition". One of the most interesting examples of the use of this system is that reported recently by Brown *et al.*[46] in which eight components of a commercially prepared anticholinergic antidote formulation, as well as an internal standard, were well separated. Other alkylsulphonates used have been the pentane[48] and the methane homologues[49,50]. Either reversed-phase[48] or straight-phase[49,50] systems can be used.

IV.1.d. Specific pairing ions

The three types of pairing ions discussed thus far, alkylammonium, alkylsulphonate and picrate, may all be considered for general-purpose use. In this section the use of various ions for more specific purposes is discussed.

One of the major advantages of ion-pair HPLC is that compounds with a low detector response can be resolved with high sensitivity by combination with pairing-ion systems having a high response. This has been demonstrated with the picrate system[19,39,40] for the separation of nanogram amounts of solute. The search for pairing ions which meet both the chromatographic requirements and which permit sensitive resolution of, for example, poorly chromophoric solutes, has continued. In two publications Lagerström and co-workers have described the use of N,N-dimethylprotriptyline[51] and N-methylimipramine[52] as suitable pairing ions incorporated in the stationary phase of a straight-phase HPLC system using ethanolyzed cellulose (Munktell 410) as stationary support. For both systems a mobile phase of *n*-hexane–methylene chloride–1-pentanol was necessary to effect the correct extraction conditions. For the N,N-dimethylprotryptyline system using detection at 254 nm and a series of carboxylic acids as model sample ions, it was demonstrated[51] that down to 15 ng of the acids could be determined with acceptable precision and accuracy. Using the N-methylimipramine system, however, detection sensitivity at 254 nm could be demonstrated to have been improved three-fold. The method can only be used when the pairing ion is eluted as an ion pair with the lowly chromophoric sample ion, thus it is ideally employed in straight-phase ion-pair HPLC. Like all straight-phase systems the pairing ion must be present in excess in the aqueous stationary phase. It can be seen from eqn. 13 that the capacity ratio of the sample ion in such systems will be controlled by both the formation of ion pairs with the highly absorbing pairing ion, and possible side-reaction mechanisms. Both ions can form both higher order aggregates between themselves and sample ions[52] and also self-association species. This will generally lead to both unstable columns and assymetrical sample peaks. However, these phenomena can be used to improve column stability because the tendency of these pairing ions to form dimers, trimers, etc. in the aqueous phase causes the concentration of the monomeric form of the ion to remain fairly constant, even when rather large alterations in the total pairing-ion concentration occur. This will give the column a high stability since bleeding will be low and thus capacity ratios will remain fairly constant even over a long period.

Naphthalene-2-sulphonate has also been used as a highly absorbing pairing ion[53–56] in the resolution of cationic ions. Early studies have been reported using either cellulose or diatomaceous earth as the stationary supports and with the sample being injected as the ion pair. Although these supports generally give rise to only moderate peak symmetry, the earlier papers[53,54] showed that this pairing-ion system could be used for the separation of alkylammonium ions having different degrees of substitution, that low pH values could be used without any disturbing bleeding and that ion pairs with a molar absorptivity of about $3 \cdot 10^3$ at 254 nm were formed. Once again it has required the introduction of more sophisticated stationary supports, such as low particle size silica of moderate pore size, to effect satisfactory chromatographic separations[56]. Naphthalene-2-sulphonate is almost aprotic and can thus be used as a pairing ion at low pH (*ca.* 2.00). This is useful for the resolution of most amines and amino acids of interest since the distribution of these solutes in their unionised form will be negligible. The system has been characterised[56] by examining the retention behaviour of a variety of amines, amino acids and peptides and comparing these results to batch extraction data. It has been found that the amino acids, the peptides and the more hydrophilic amines required a pairing-ion stationary phase concentration of 0.1 mol·dm^{-3} for suitable retention to be obtained; additionally for these solutes good correlation between batch extraction data (partition) and chro-

matographic behaviour could be shown. With the more hydrophobic amine ions studied, however, the agreement between the found capacity ratios and those calculated from batch extraction $E_{A,B}$ values was poor, and for such hydrophobic amines lower pairing-ion concentrations (0.01 mol·dm^{-3}) were needed. These authors conclude that ion-pair distribution is not the predominant factor in the retention of those more hydrophobic amines, and largely base this conclusion on the rather small difference in extraction constants (about 0.2 log units) found between *n*-butylamine and *n*-octylamine extracted as naphthalene-2-sulphonate ion pairs into chloroform–1-pentanol, although this latter observation is a surprising one.

IV.2. Inorganic ions

The first literature reference to the use of pairing ions in column chromatography came with the work of Wahlund and Groningsson[57] who presented methods for the reversed-phase "partition" chromatography of organic ammonium compounds as ion pairs with inorganic anions. Silicone-treated acetylated cellulose was used as the support, lipophilic alcohol mixtures as the adsorbed stationary phase and aqueous solutions of inorganic ions as the mobile phase. Although, with perchlorate in the mobile phase, these workers were able to attain reasonable separations of drugs such as papaverine and strychnine, with the primitive supports used poor column efficiencies were found. Other earlier studies[10,58] reported the same problem. The use of stable non-compressible stationary supports subsequent to those earlier studies has led to a remarkable improvement in column separation efficiencies for inorganic ion-pair HPLC separations.

The first consideration with use of these inorganic pairing ions is that their extremely high hydration will prohibit ion pairing in environments of high dielectric constant (section II), and that such association species will thus be formed in the diffusional or interfacial layers between phases. Inorganic ions used as HPLC pairing ions have been perchlorate, bromide, chloride, nitrate, sulphuric, citrate and phosphate. Table 3 lists their use together with the phase systems and solutes examined.

IV.2.a. Perchlorate

The use of perchlorate as a pairing ion has been developed from its successful use for ion-pair bulk-phase extractions[7]. For HPLC purposes a variety of stationary phases, supports and eluents have been examined. For example, Borg *et al.*[35] have studied the drug alprenolol and its metabolites in a plasma sample using cyclohexane–1-pentanol (93:7) as mobile phase and an ethanolyzed cellulose support coated with an aqueous phase of 0.9 mol·dm^{-3} $NaClO_4$ in 0.1 mol·dm^{-3} $HClO_4$. Owing to the high separation factors attained with this system the column's efficiency was sufficient for the separation of alprenolol and two of its metabolites N-desisopropylalprenolol and 4-hydroxyalprenolol. Bulk-phase extraction data had shown that dissimilar solvation of the drug compared to its 4-hydroxy metabolite occurred with 1-pentanol as part of the extracting phase. Thus for the ion-pair HPLC determination of 4-hydroxyalprenolol alone, cyclohexane–1-pentanol (80:20) produced good separation. It is likely that these workers would have achieved a greater selectivity had halogenated hydrocarbons[19] been used as part of the eluent, although their use with the liquid scintillation assay employed would have produced quenching effects.

Knox and Jurand[59] similarly reported on the use of a 0.1 mol·dm^{-3} $HClO_4$–0.9 mol·dm^{-3} $NaClO_4$ combination as the stationary aqueous phase using the more preferable non-compressible stationary support Merckosorb SI 100 silica gel. With

no complicating assay these workers were able to use the indicated halogenated hydrocarbons as eluent components, and examined either chloroform or methylene chloride with aliphatic alcohols as eluents. Using cationic drugs, including some phenothiazines and dibenzazepines (Table 3), as model solutes, it was found that capacity ratios decreased as the percentage of alcohol in the eluent increased; and that an increase in the alcohol alkyl chain length caused the capacity ratios to increase (*i.e.* 1-butanol to isopentylalcohol caused a 1.4- to 2-fold increase in capacity ratios). Conversely a substitution of chloroform by methylene chloride in the eluent caused a 1.4- to 2.5-fold decrease in capacity ratios. Although it was argued that these effects are in the direction expected on the basis of concurrent changes in the eluent's Snyder solvent strength parameter[60], they can also be argued for on the basis of specific solvating mechanisms[3,4,19]. A brief attempt was also made in this study to rationalise the effects of sample structure on retention, and it was found that a central heterocyclic group of the drug's fused ring system had a major influence on retention and that for the phenothiazine and dibenzazepine series studied the order of elution was strongly influenced by the nature of a side chain attached to the central nitrogen atom. It was concluded that this ion-pair chromatographic system could be most effective when a group separation is required, and that the main discriminating factor is the number and basicity of the nitrogen atoms in the compounds.

A priori the effect of the nature of the stationary support using perchlorate or other inorganic ions as the stationary phase would be expected to be a significant one. So it is interesting that separate studies[25,32,33] using perchlorate coated onto silica supports of widely different surface areas have indicated that the relative retentions of some biogenic amines are independent of the silica surface areas. This shows that the solid surface does not contribute to retention even on the higher-surface-area supports (although the suggestion[32] that this phenomenon may therefore be related to the fact that the stationary phase consists mainly of water, and that a controlling factor in the distribution process is the secondary chemical equilibrium of ion-pair extraction, is somewhat confusing).

Relative retention in these ion-pair systems may be defined as:

$$r_{ji} = \kappa_j/\kappa_i = E^*_{j,B^-}/E^*_{i,B^-} \tag{25}$$

where r_{ji} is the selectivity factor, subscripts j and i refer to later and earlier eluted species, and B^- is the extracting pairing ion. Using silica supports Karger *et al.*[33] have optimised the perchlorate system by using highly solvating components diluted with hexane as the mobile phase. By examining r_{ji} values good separations for primary and secondary biogenic amine pairs could be shown to occur using relatively weak solvating components such as 1-butanol and ethyl acetate in the eluent. However, for the primary and secondary amine pairs normetanephrine–metanephrine and noradrenaline–adrenaline little separation could be achieved until a very basic organic phase modifier (tributyl phosphate) was used. With such a system r_{ji} values between these solute pairs rose to *ca.* 1.8, which was attributed to the greater hydrogen-bonding capacity of the primary amine, resulting in enhanced solvation of the formed perchlorate ion pair and thus elution of the primary amine before the secondary amine. Once again this is an example of specific solvate formation being used to regulate solute retention. It should be appreciated, however, that solvate-forming components should be selected with some care; for example tributyl phosphate which works so well in the separation of the primary and secondary amines, gives rise to

a very low sensitivity for the corresponding hydroxy and methoxy compound, and it has been argued[25] that the possible strong acid–base interactions expected between phosphate and phenolic groupings minimise any differences in intramolecular hydrogen bonding that could arise in the catecholamines and their methoxy derivatives. Interestingly a combination of two solvating agents, ethyl acetate and tributyl phosphate, in hexane can effect the separation of the series of eight biogenic amines studied. In addition, and as found by other workers[59], the use of a halogenated hydrocarbon (methylene chloride) leads to a greater extraction of the amino–perchlorate ion pair relative to a hexane system. This effect has been discussed previously (section IV.1.b) for the picrate system.

Eqns. 10–13 show that there should be a relationship between retention in an ion-pair HPLC system and bulk phase solvent extraction parameters. Fig. 4 has been constructed from data taken from ref. 33 and relates distribution data for a series of biogenic amines obtained both chromatographically and by bulk-phase extractions, using a perchlorate pairing ion in both cases. The relationship between the two sets of distribution data is embodied in

$$D_{HPLC} = 0.90\, D_{bulk} + 0.26 \qquad (26)$$

The derived relationship has a correlation coefficient, r, of 0.997 which implies that *ca.* 1.0% of the variance in the correlation is unexplained by the relation. Such a relationship suggests that ion-pair distribution is the prime retention mechanism in the HPLC system and that side-reactions are not significant. The same authors[33], however, indicate that such correlation between HPLC and bulk-phase extraction

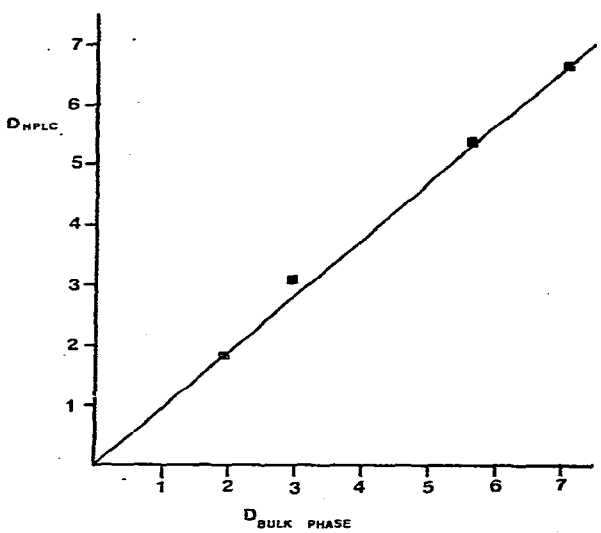

Fig. 4. Relationship between distribution ratios for amine–perchlorate ion pairs measured by both HPLC and bulk-phase extraction. Chromatographic conditions: stationary phase, 43% (w/v) loading of 0.2 mol·dm^{-3} $HClO_4$–0.8 mol·dm^{-3} $NaClO_4$; mobile phase, butanol–methylene chloride (2:3); stationary support, Merckosorb SI 100 (surface area 250 m^2·g^{-1}); temperature, 20°. Bulk-phase solvent extraction conditions: aqueous phase, 0.2 mol·dm^{-3} $HClO_4$–0.8 mol·dm^{-3} $NaClO_4$; extracting phase, butanol–methylene chloride (2:3); temperature, 25°.

data will not always exist owing to both experimental problems, *e.g.* phase mutual saturation, and secondary interfering equilibria.

Table 3 lists other studies which have used the perchlorate system. There appear to be two major disadvantages with its use. First, there is the problem of column instability caused by a bleeding or stripping of the stationary phase. The only satisfactory answer[61] to this problem would appear to be the use of a stationary support adsorbent having a large surface area to extract a polar component from a mixed mobile phase (*e.g.* hexane–acetonitrile, 99:1). In essence this would result in a two-phase system stabilized by the presence of the adsorbent surface. The difficulty which arises with this approach for ion-pair HPLC is the high buffer salt concentrations generally present in the stationary phase. Other workers[19] have suggested that so long as the pairing ion is in large excess the problem of bleeding only becomes apparent after considerable use.

A second problem with the perchlorate system is one which is often reported[19,33,57] and that is significant sample peak tailing. Peak symmetry has been shown[50] to be improved with the perchlorate ion-pair HPLC systems using *n*-hexane as eluent, by the addition of amounts of 1-butanol ($\geqslant 5\%$) to the mobile phase. It will also be shown in section IV.3.b that the addition of competing pairing ions to perchlorate systems also results in improved peak symmetry.

The utilization of specific solvating processes and competing equilibria in straight phase ion-pair HPLC has been developed to a sophisticated level by Kraak and Huber[62] who have examined the separation of acidic compounds by the use of an aqueous perchloric acid (mobile phase)—tri-*n*-octylamine (stationary phase) system. They were able to show that efficient separations could be obtained when either diatomite (5–10 μm) or low-surface-area silica (Spherosil XOC 005) was the support material. In their study perchlorate solutions of 0.05 mol·dm^{-3} having different pH values were used. Kraak and Huber[62] were able to write a simple formula which shows the dependence of the solute distribution coefficients on single equilibria constants, pH and ion concentration in the aqueous phase. If one considers the total distribution of an acid HX between an aqueous phase containing a strong acid HB and an organic phase consisting of a long-chain aliphatic amine A (*i.e.* tri-*n*-octylamine) then the following equilibria may be considered.

(1) Liquid–liquid distribution of the undissociated acid HX between the stationary and the mobile phases:

$$HX_{aq} \rightleftharpoons HX_{org}$$

$$D_{HX} = \frac{[HX]_{org}}{[HX]_{aq}} \tag{27}$$

where D_{HX} is the distribution ratio of HX.

(2) Dissociation of the acid HX in the mobile phase:

$$HX_{aq} \rightleftharpoons H^+_{aq} + X^-_{aq}$$
$$K_1 = [H^+]_{aq} \cdot [X^-]_{aq} \cdot [HX]^{-1}_{aq} \tag{28}$$

where K_1 is the dissociation constant of HX.

(3) Exchange of the anion X^- in the mobile phase with the ion pair AHB in the stationary phase:

$$X^-_{aq} + (A,HB)_{org} \rightleftharpoons (A,HX)_{org} + B^-_{aq}$$
$$K_2 = [A,HX]_{org} \cdot [B^-]_{aq} \cdot ([X^-]_{aq} \cdot [A,HB]_{org})^{-1} \quad (29)$$

where K_2 is the ion-exchange constant for X^- in the mobile phase and the ion pair A,HB in the stationary phase.

(4) Ion-pair formation between the dissociated acid HB in the mobile phase and the amine A in the stationary phase:

$$A_{org} + H^+_{aq} + B^-_{aq} \rightleftharpoons (A,HB)_{org}$$
$$K_3 = [A,HB]_{org} \cdot ([A]_{org} \cdot [H^+]_{aq} \cdot [B^-]_{aq})^{-1} \quad (30)$$

where K_3 is the formation constant of the ion pair A,HB in the organic stationary phase.

(5) Exchange of the proton of the acid HX in the stationary phase for the cation M^+ in the mobile phase which is assumed to be a constituent of the eluent.

$$M^+_{aq} + HX_{org} \rightleftharpoons MX_{org} + H^+_{aq}$$
$$K_4 = [MX]_{org} \cdot [H^+]_{aq} \cdot ([HX]_{org} \cdot [M^+]_{aq})^{-1} \quad (31)$$

where K_4 is the ion exchange constant for the cation M^+ in the mobile phase and the acid HX in the stationary phase.

Since it is assumed that A and (A,HB) are insoluble in the mobile phase and also that the sample is present in low concentration, the overall concentration C of the stationary phase (which is constant) may be given by:

$$C = [A]_{org} + [A,HB]_{org}$$

Thus the total distribution coefficient of X is given by:

$$D_X = \frac{[HX]_{org} + [(A,HX)]_{org} + [MX]_{org}}{[HX]_{aq} + [X^-]_{aq}} \quad (32)$$

From eqns. 26–31 Kraak and Huber were able to derive an expression (eqn. 32) that describes the total distribution as the sum of three terms:

$$D_X = D^1_X + D^2_X + D^3_X \quad (33)$$

where

$$D_X{}^1 = D_{HX} \cdot \frac{1}{1 + K_1 \cdot [H^+]^{-1}_{aq}}$$

$$D_X{}^2 = K_2 \cdot C \cdot \frac{1}{([B^-]_{aq} + (K_3 \cdot [H^+]_{aq})^{-1}) \cdot (1 + [H^+]_{aq} \cdot K_1^{-1})}$$

$$D_X{}^3 = D_{HX} \cdot K_4 \cdot \frac{[M^+]_{aq}}{[H^+]_{aq} + K_1}$$

Thus eqn. 33 shows the dependence of the total distribution on the concentrations of the eluent anion and cation and the pH of the mobile phase, with the terms $D_X{}^1$ describing the distribution of the acid, $D_X{}^2$ the effect of ion-pair formation with tri-*n*-octylanine and $D_X{}^3$ the effect of ion-pair formation with an eluent cation.

These workers investigated the effects of temperature, perchloric acid con-

centration, pH and concentration and nature of salt added to the mobile phase. The validity and usefulness of eqn. 33 for this system can be shown by consideration of the first of these factors, *i.e.* temperature. Choosing conditions such that D_X^3 was zero, it was found that the change in capacity ratio with temperature (25°–65°) was insignificant for some acids but highly significant for others in which the capacity ratio can either decrease or increase. If D_X^1 is dominant, then D_X will to a large extent be dependent on D_{HX}; conversely, if D_X^2 predominates then the ion-exchange constant K_2 will determine the temperature dependence of the total distribution. Assuming that D_{HX} decreases and K_2 increases with temperature, it has been concluded[62] that for phenols and carboxylic acids at pH 1.5 liquid–liquid distribution is the major process since their capacity ratios decrease with temperature. Similarly for a sulphonic acid series where capacity ratios increased with temperature it was concluded that ion-pair formation is the major process at pH 1.5.

IV.2.b. Halides

The use of small inorganic ions for preparative ion-pair HPLC has been partially indicated by the sucessful separation[63] of the quaternary ammonium ion emepronium from its ring substituted chloro analogues, using silicone treated ("hydrophobized") cellulose as stationary support, 1-pentanol as stationary phase (pre-equilibriated with the mobile phase) and aqueous solutions of bromide or chloride as the mobile phase. Samples were injected initially as either the bromide or chloride salt, depending on the mobile phase used (*i.e.* either 0.07 mol·dm^{-3} NaCl or 0.02 mol·dm^{-3} NaBr). At low sample loadings good agreement existed between bulk-phase extraction parameters and chromatographic retention, but at higher loadings (section III.3) of sample, such that the sample's counter ion was significantly contributing to the pairing ion's total concentration, retention volumes increased. This phenomenon was observed especially with the bromide system.

The effect of adding large quantities of bromide ion into a chloride–1-pentanol system was also investigated. When a small amount of *p*-chloroemepronium was applied as the bromide salt, a double sample peak (Fig. 5) was observed. This was due to column retention of both chloride and bromide ion-pairs. At even higher bromide concentrations, however, peak overlapping resulted in one broad band only.

If such double peaks and competing ion-pair equilibria are found in practice, then compensations have to be made in the description for solute retention. Thus, for example, with the emepronium bromide salt in a chloride system, compensations

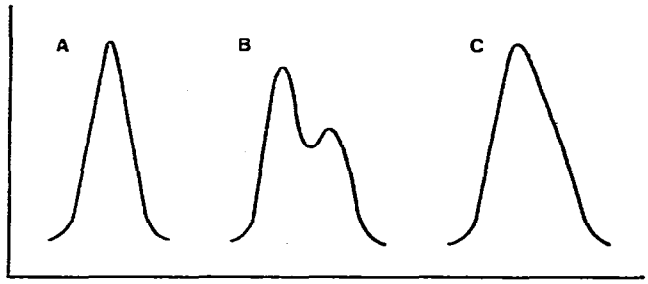

Fig. 5. Idealised chromatograms showing the influence of counter-ion type on the chromatography of a solute ion S with a pairing ion Y. (A) Sample injected as the SY salt form, resulting in retention as the S,Y ion pair. (B) Sample injected at low concentration as the SX salt form, resulting in double peaks due to retention as both S,Y and S,X ion pairs. (C) Sample injected at high concentrations as the SX salt form, resulting in a broad peak due to overloading with the S-X ion pair.

need to be made for the distribution of bromide–emepronium ion pairs. That is, the total sample (A) distribution is given by:

$$D_A = E^*_{A,Cl} \cdot C_{Cl^-} + E^*_{A,Br} \cdot C_{Br^-} \quad (34)$$

(*cf.* eqn. 7) Dissociation of the bromide–sample ion pairs can occur[63] in the organic (*i.e.* stationary) phase, thus:

$$C_A = [A^+]_m + [A,Br]_s + [A,Cl]_s + [A^+]_s \quad (35)$$

$$C_{Br} = [Br^-]_m + [A,Br]_s + [Br^-]_s \quad (36)$$

where C_A and C_{Br} are the total concentrations of these ions and subscripts m and s refer to mobile and stationary phases. Eqns. 35 and 36 are equal since emepronium is added as the bromide salt. Assuming that chloride ion pairs do not dissociate, then: $[A^+]_s = [Br^-]_s$, and thus

$$D_A = (E^*_{A,Cl} \cdot C_{Cl}) + E^*_{A,Br} \cdot C_{A^+} \cdot [1 + E^*_{A,Cl} \cdot C_{Cl}] + (k_{diss(A,Br)} \cdot E^*_{A,Br} \cdot [1 + E^*_{A,Cl} \cdot C_{Cl}])^{1/2} \quad (37)$$

Fig. 5 diagrammatically illustrates this effect.

IV.3. Mixed systems

In all the studies thus far considered the pairing ion has been a single ion. There are times, however, when a combination of pairing ions, either used concurrently or consecutively, can be indicated.

IV.3.a. Indicating pairing ions

The often favourable separations possible using inorganic ions such as chloride (section IV.3.b) as the pairing ion, are often wasted if the sample to be resolved is a non-chromophore. In such cases highly chromophoric pairing ions are necessary although the then resultant $E_{A,B}$ values may be now too high etc. To meet this problem of good chromatographic separation, for example with chloride ions, but insensitive detection owing to a poorly chromophoric sample Eksborg[55] has presented methods for the formation of highly UV-absorbing derivatives by the initial isolation of a non-chromophoric sample as a non-chromophoric ion pair with chloride in a "separating" column, which is then exchanged for a highly UV-adsorbing ion in a small column (the "indicating" column) situated between the end of the separating column and the detector (Fig. 6).

Using alkylammonium ions (A) as samples, chloride as separating pairing ion, naphthalene-2-sulphonic acid (section IV.1.d) as indicating pairing ion, Dia-Chrom (particle size 37–44 μm) as support and chloroform–1-pentanol (19:1) as mobile phase, Eksborg[55] has developed a system which gives almost symmetrical peaks and where the indicating column does not significantly decrease the separating efficiency of the chloride column. Eksborg points out that bleeding of the indicating pairing ion can cause disturbances in the transport of the sample ion through the column unless another pairing ion with sufficiently high extraction ability is present in the indicating column. To avoid such disturbances the stationary phase of the indicating column needs to contain a mixture of the indicating ion (X^-) and the separating ion (B^-). Eksborg has stated that for a quantitative exchange of separating

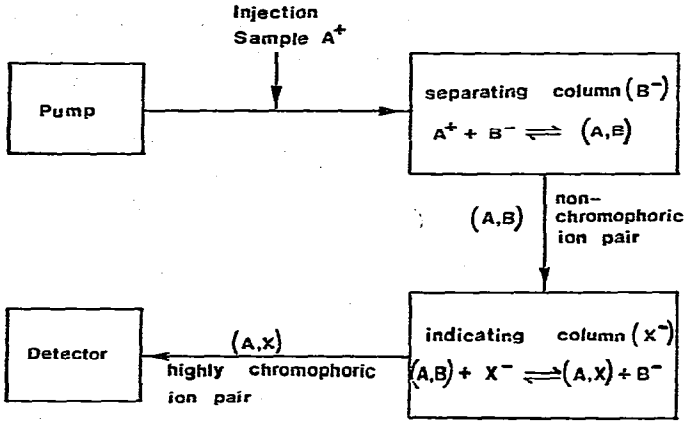

Fig. 6. Flow diagram illustrating the technique of using a separating and an indicating column[55] where B^- and X^- are separating and indicating pairing ions, respectively (see section IV.3.a.)

to indicating pairing ion the following requirement is necessary in the indicating column:

$$E^*_{A,X} \cdot C_{X^-} \geqslant 100\,(E^*_{A,B} \cdot C_{B^-}) \tag{38}$$

For a chromatographic system containing a separating and indicating column and assuming the capacity ratio in the separating column to be approximately zero, then

$$\kappa_A = (V_R - V_{mi}) \cdot V_{ms}^{-1} - 1 \tag{39}$$

where V_{mi} and V_{ms} are the interstitial volumes of indicating and separating columns and where V_R is the retention volume. The technique, although an attractive one, needs to be examined in greater detail before it can be regarded as a general procedure, and it would appear preferable at this stage to use a single pairing ion of high chromophoric nature and correct extraction parameter for resolving poorly chromophoric solutes.

IV.3.b. Secondary equilibria suppression

By far the most significant problem with ion-pair HPLC is the commonly observed assymmetry or tailing of sample peaks. This is generally attributable to competing secondary equilibria such as dissociation in the organic phase or dimerization of the pairing ion etc. A number of studies[19,21,27,41,42,53,55,64–68] have attempted to deal with this problem not by modifying the extracting phase, (section IV.2.a), but by using combinations of pairing ions.

Eqn. 6 shows how the influence of side-reactions on an ion-pair distribution can be handled using conditional extraction constants, where the α-coefficients include all side-reaction effects. Association or dissociation processes will vary of course with the concentration of the migrating sample and, unless suppressed, will result in asymmetric peaks or other deviations from accepted chromatographic behaviour. Thus small amounts of samples sometimes[19] give rise to leading peaks which may be attributable[53] to dissociation in the organic phase, the degree of dissociation varying with the nature of the ion pair. For example, quaternary ammonium ion pairs gener-

ally have higher dissociation constants than amine ion pairs[68]. The dissociation will also depend on the polarity of the organic phase, with methylene chloride and 1-pentanol, for example, resulting in higher ion-pair dissociation than with chloroform and hexane[18] (section IV.1.b).

Picrate columns often give rise to tailing of sample peaks and disturbances in capacity ratios. An increase in capacity ratio with straight-phase systems could well be due to too high sample concentrations leading to an increase in the number of ion-pair associations in the aqueous phase, so that an increase in sample concentration will lead to an increase in capacity ratio. A decrease in sample capacity ratio can be due to either formation of dimeric or polymeric picrate ion pairs in the organic phase, or to a significant increase in the concentration of picrate in the aqueous phase, or to dissociation of the picrate–sample ion-pair in the organic phase. Dissociation constants for quaternary ammonium–picrate ion-pairs have been found to be 1×10^{-4} in methylene chloride[18] and estimated[11] to be 1×10^{-6} in chloroform.

The disturbances by ion-pair dissociation increase therefore when the sample concentration is low. It can be shown experimentally that the addition of hydrophobic ions in sufficient excess will completely suppress the dissociations of, for example, picrate–sample or alkylammonium–sample ion pairs, and thus lead to reproducible peak shape and position. Continuing with the picrate example, if the mobile phase contains ion pairs of picrate (P) with sample (B^+) and with added hydrophobic cation (A^+), then the following equations may be written:

$$[P]_m = [A]_m + [B]_m \tag{40}$$

$$K_{diss(P,A)} = [P]_m \cdot [A]_m \cdot [P,A]^{-1} \tag{41}$$

$$K_{diss(P,B)} = [P]_m \cdot [B]_m \cdot [P,B]^{-1} \tag{42}$$

$$E_{P,A} = [P,A]_m \cdot ([P] \cdot [A])^{-1} \tag{43}$$

$$E_{P,B} = [P,B]_m \cdot ([P] \cdot [B])^{-1} \tag{44}$$

The dissociation process will affect E^* by $\alpha_{P,A}$ (see eqn. 6) such that:

$$\alpha_{P,A} = ([P,A]_m + [A]_m) \cdot [P,A]_m^{-1} = 1 + (K_{diss(P,A)} \cdot [P]_m^{-1}) \tag{45}$$

Eqns. 40–45 may be combined[64] to give:

$$[P]_m = (K_{diss(P,A)} \cdot E_{P,A} \cdot [P] \cdot [A] + K_{diss(P,B)} \cdot E_{P,B}\,[P] \cdot [B])^{1/2} \tag{46}$$

or by grouping constants:

$$[P]_m = [P]^{1/2} \cdot (a \cdot [B] + b)^{1/2} \tag{47}$$

It can now be seen that if the term $(a \cdot [B])$ is very much greater than b, then $[P]_m$ will vary with the sample concentration. The sample concentration will decrease during the migration process and will give rise to a decrease in capacity ratio (assuming that the change in $[P]_m$ has a significant influence on $\alpha_{P,A}$); such an effect will also manifest itself in the sample peak having a loading front. It thus follows that no such effect will be seen when the constant b is very much greater than the term $(a \cdot [B])$. The presence of a secondary hydrophobic cation B^+ in sufficient concentration will therefore influence the b term such that this second condition will

result in symmetrical, reproducible peaks.

For reversed-phase systems disturbances in peak shape and position may also be minimised by addition of additional ions. If A^+ (the pairing ion) can be similarly kept at a constant level then from eqn. 21 it follows that the conditional extraction constant $E^*_{A,B}$ will not change with a change in sample concentration. Again this may be achieved if A^+ is also extracted by the organic stationary phase as an ion pair with a anion (Z^-) as well as with the sample ion (B^-). For this case if both ion pairs (*i.e.* A,Z and A,B) have similar dissociation constants and if the concentration of the pairing ion–added anion ion pair (*i.e.* A,Z) is very much greater than that of the pairing ion–sample anion ion pair, then the amount of pairing ion in the organic phase (*i.e.* $[A^+]_{org}$) will be independent of the concentration of A,B in the organic phase. The conditional extraction constant will now be given by[27]

$$E^*_{A,B} = E_{A,B} \cdot (1 + K_{diss(A,B)} \cdot (K_{diss(A,Z)} \cdot E_{A,Z} [A^+] \cdot [Z^-])^{-1/2}) \quad (48)$$

If the concentrations of pairing ion and added ion are kept constant in the mobile aqueous phase, then from eqn. 48 it follows that $E^*_{A,B}$ will be independent of the concentration of the sample and hence symmetrical peaks will result.

Wahlund[27] has shown that there are a number of observations which indicate that the dissociation of tetrabutylammonium–benzoate ion-pairs in reversed-phase systems can be suppressed by the addition of inorganic anions, such as $H_2PO_4^-$ or SO_4^{2-}, which ion-pair with the alkylammonium ion. Similarly it is pointed out that the capacity ratio of toluene-4-sulphonate on a reversed-phase column, using tetrabutylammonium ion in 0.04 mol·dm^{-3} phosphate buffer as mobile phase falls if the mobile phase is adjusted to 0.1 mol·dm^{-3} with respect to bromide. This is suggested[27] as being due to the decrease in $E^*_{A,B}$ (eqn. 48) by bromide ions which have a much higher $E_{A,Z}$ than phosphate[69]. Examples of added ions to suppress dissociation effects include the use of phosphate buffer ions in tetrabutylammonium columns for the analysis of mandelic and vanilmandelic acids[66], and the addition of tetraethylammonium chloride to a straight-phase chloride pairing-ion system[55] for the efficient resolution of tricyclic antidepressant drugs.

IV.3.c. Displacement of disturbing pairing ions

The analysis of many drugs and metabolites by ion-pair HPLC very often involves the use of a pre-extraction procedure to concentrate the solute from a possible complex environment. An obvious means by which this can be achieved is by ion-pair extraction[1,2] into an organic bulk solvent. The pairing ions used for this step often have a high hydrophobic integrity, and hence have high $E_{A,B}$ values. Figs. 1 and 2 show that for many ion-pair HPLC systems a low $E_{A,B}$ is often needed. Injection of an extracted solute and its extracting pairing ion into an ion-pair HPLC system can thus mean that present in the system is an additional pairing ion having a high extracting ability. The result of this can be a disturbance in the chromatography of the solute owing to a discontinuous distribution process. Such effects have been reported with the chromatography of acetylcholine and choline extracted from brain homogenates using hexanitrodiphenylamine (HNP)[41,42] and studied using a picrate straight phase column. At the pH of this system (6.5) the extracting ion (HNP) will travel both as the acid and the HNP–acetylcholine (or choline) ion pair. The acid HNP species will increase in concentration continuously during its passage through the column which results in incomplete resolution. The addition of an excess of a more hydrophobic cation relative to acetylcholine (such as tetrabutylammonium) as

the picrate will result in HNP migrating now as an HNP–tetrabutylammonium ion pair, so eliminating the disturbance, and as an additional benefit, picrate will also pair with the tetrabutylammonium ion and thus (section IV.3.b) will prevent any possibility of picrate loss from the column by the elution of the sample. Recently Eksborg and Schill[67] have presented methods for the displacement of disturbing pairing ions based on theoretical considerations. Although that treatment is outside the scope of this present contribution, it is relevant to note that using their methods the computation of disturbing ion displacement conditions can be made with reasonable confidence. They also point out that the concentration of displacing ion necessary to displace the disturbing extracting ion on the column increases with (a) the product $(N^{-1/2} \cdot V_m)$, where N is the number of theoretical plates and V_m the interstitial volume, (b) increasing sample concentration and (c) increasing extracting ability of the displacing ion. This last finding, the authors point out, means that there is no advantage in using a highly hydrophobic ion as the displacing ion.

IV.4. Surface-active ions

The use of hydrophobic pairing ions such as tetramethylammonium and picrate and of hydrophilic ions such as chloride and perchlorate is somewhat overshadowed by the often found effects of tailing, fronting, peak position variability, and, especially with straight-phase systems, column instability. These effects have been discussed previously. Knox and co-workers[61,70,71] and Haney and co-workers[21,72] in an apparently independent way introduced the concept of adding hydrophobic pairing ions having long alkyl chains to the phase system. In a study on the effect of selected amine salts on the retention behaviour of tartrazine and its intermediates, Haney's group[21] used small quantities of quaternary and tertiary amines in reversed-phase systems. Apart from short alkyl chain amines (section IV.1.a) they studied tridecylamine ($3 \cdot 10^{-4}$ mol·dm^{-3}) and tridecylamine–tributylamine mixtures (3×10^{-4}:$3 \cdot 10^{-3}$ mol·dm^{-3}) and found that they could achieve excellent separation of the tartrazines. They argued, without giving complete evidence, that the mechanism of separation could be the reversible formation of ion pairs within the chromatographic system, and separation of the constituents on the basis of differences in the lipophilicities of the ion pairs so formed.

Knox and Laird[70] were at this time examining different types of HPLC methods for maximum possible efficiencies to see whether improvements in the HPLC performance could be brought about, and especially to examine whether the often necessary pre-extraction step employed with biological fluid examination could be avoided. Their work arose out of the problems encountered in the separation of various naphthalenesulphonic acid derivatives by straight-phase HPLC, and the very strong polarity of the sulphonate group causing tailing effects owing to its ability to displace water from the silanol groups of their support. It seemingly occurred to these workers that this problem might be solved by employing a pairing ion which might be expected to form highly stable ion pairs and would thus not dissociate in the presence of strongly hydrogen-bonding surface groups. Their choice of cetyltrimethylammonium bromide (CTAB) as pairing ion, and an aqueous alkaline eluent was an excellent one and satisfied their criteria; unfortunately their use of the term "soap" chromatography to describe their approach was not. Soaps are by convention long-chain carboxylic acid salts of the alkali metals. The term "detergent" HPLC which is also used[73] is incorrect, since the surface-active molecules are not necessarily acting as detergents. We have used the term "surfactant" HPLC[74], but even this is

not completely satisfactory when one considers the mechanisms of retention (which will shortly be discussed) in these systems.

Knox and Laird found in their initial experiments that while the addition of *ca.* 1% CTAB improved the adsorption chromatography of the acids on silica gel, the system could not be well defined. Thus they turned to a reversed-phase support (Wolfson SAS silica) in which the silica surface is covered by short alkylsilyl groups bonded to the surface by Si–C bonds. Very importantly, and in contrast to Haney's publications, these workers expected the surfactant to be directly adsorbed by the support, and also anticipated that this would render the dependence of sample retention on CTAB concentrations to be more readily interpretable.

Using a series of dyestuffs and intermediates they showed that with reversed-phase systems an increase in CTAB concentration up to approximately $7 \cdot 10^{-3}$ mol·dm^{-3} in the eluent increased the capacity ratios; above this concentration of CTAB a subsequent fall in capacity ratio was noted, although the order of elution was unaffected. Knox and Laird explain these phenomena by supposing that with low concentrations of CTAB three physicochemical processes occur. That is, formation of CTAB–sample ion-pairs, distribution of these ion pairs to the stationary hydrophobic phase and adsorption of CTAB via hydrophobic interactions onto the surface of the reversed-phase support. They present these equilibria by Scheme 3:

$$\begin{array}{ccc} \underline{nA^+_{aq}} + S^{n-}_{aq} & \rightleftharpoons & \underline{(nA,S)_{aq}} \\ \alpha \Big\downarrow & K_{aq} & \\ \underline{nA^+_{ads}} + S^{n-}_{ads} & \rightleftharpoons & \underline{(nA,S)_{ads}} \end{array} \qquad \beta \Big\downarrow$$

Scheme 3.

where the subscripts aq and ads refer to the eluent and interface phases, respectively, and where A and S are cetyltrimethylammonium and sample ions, respectively, and where underlined species are considered to be present in high relative concentrations. By considering column breakthrough times for the pairing ion it was shown that indeed CTAB is adsorbed by the surface, with its adsorption isotherm being curved over a $0–5 \cdot 10^{-2}$ mol·dm^{-3} CTAB concentration, and obeying a simple Freundlich-type equation, *i.e.*:

$$[A^+]_{ads} = \alpha[A^+]^{0.8}_{aq} \tag{49}$$

They calculated that this means that at a $5 \cdot 10^{-2}$ mol·dm^{-3} CTAB concentration using their support material, surface coverage of CTAB cation is between 5 and 15%, depending on the "depth" assigned to the alkyl chain on the surface. Such a low coverage is taken to assume that a considerable part of the surface must be covered by adsorbed aliphatic alcohol (1-propanol) from the eluent. By assuming that the concentration of sample ion $[S^{n-}]_{ads}$ in the stationary phase is negligible compared to that of the A,S ion pair it can be derived[70] that the distribution coefficient D for sample between the stationary and eluent phase is:

$$D = \frac{\alpha^n \cdot \beta \cdot K_{ads}\,[A^+]^{0.8n}_{aq}}{1 + K_{aq} \cdot [A^+]^n_{aq}} \tag{50}$$

where K is the formation constant of the (A,S) ion pair in each phase. Eqn. 50 shows that at low CTAB concentration the capacity ratios will rise (*cf.* eqn. 9). The fall in capacity ratio with increasing CTAB concentration has been attributed to either CTAB micelle formation or at least to CTAB ion clusters which solubilize the sample ions (and in fact act partly as a detergent in this mode).

The dependence of dyestuffs' and intermediates' capacity ratios on CTAB concentration does not show the same effect using silica gel as the stationary support. The observed trend is for the degree of retention to increase with increase in CTAB concentration, although the rate of increase falls at higher surface active agent concentrations. Since no evidence of pairing-ion adsorption onto silica gel was found, the authors conclude from many experimental observations that sample retention with straight-phase systems using surface-active pairing ions is one in which (A,S) ion pairs are present in the eluent and are solvated by the aliphatic alcohol (1-propanol) present. These solvated ion pairs are then adsorbed most readily onto a surface which can itself be heavily solvated by 1-propanol, so that the 1-propanol molecules act as a "binder" to hold lipophilic ion pairs onto the hydrophilic silanol surface. Also there is no involvement of micellar or clustered CTAB species.

Notwithstanding these theoretical considerations, the method of using CTAB appears to be satisfactory and has recently[75] been applied to the determination of the α,β-adrenoreceptor antagonist labetalol in biological fluids, although a pre-extraction step was considered necessary.

For the separation of cations such as catecholamines, Knox and Jurand[61] and Jurand[71] have used long-chain alkylsulphates as pairing ions using reversed-phase systems. The alkylsulphates used include sodium dodecylsulphate, sodium 1-dodecanesulphonate and sodium dodecylbenzenesulphonate. The concentrations of surface active agent used, however, were between 0 and 1 %, and the order of sample elution was approximately the reverse for that found using straight-phase adsorption and simple ion-pair (perchlorate) HPLC. Up to 1 % it was found that relationships between capacity ratios and surface-active ion concentrations were similar to those found using the CTAB system. We have recently performed experiments along similar lines to those of Knox and Jurand, although an homologous series of alkylsulphates has been used as surface-active pairing ions. Fig. 7 illustrates our findings using tryptophan as the model solute. Three effects can be noted. First that there is an increase in capacity ratio of tryptophan with increasing pairing-ion concentration, second that for an equivalent concentration the capacity ratio is higher the longer the alkyl chain of the pairing ion, and third that there is a sigmoidal relationship between capacity ratio and pairing-ion concentration. Similarly Gloor and Johnson[23] have used alkyl sulphonates for the separation of biogenic amines.

Horváth and co-workers[76,77] have recently presented extensive experimental evidence, and a theoretical treatment based on solvophobic theory, showing that the mechanism of sample retention with "soap" (sic) chromatography is ion-pair formation with the surfactant pairing ion in the mobile phase resulting in a higher capacity ratio of sample as the ion pair compared to the non-paired species. They report that decylsulphate at pH 2.1 is suitable for the separation of amino acids using a LiChrosorb RP-18 (5 μm) column support and eluent of 0.1 mol·dm^{-3} phosphate buffer containing $3 \cdot 10^{-3}$ mol·dm^{-3} sodium decylsulphate at 70°. An awareness of the incomplete elucidation of retention mechanisms with surface-active ion-pair HPLC, combined with experience[24] of ion-pair formation between the cationic surface-active agents —alkylbenzyldimethylammonium chlorides (ABDACs)— and anionic drug

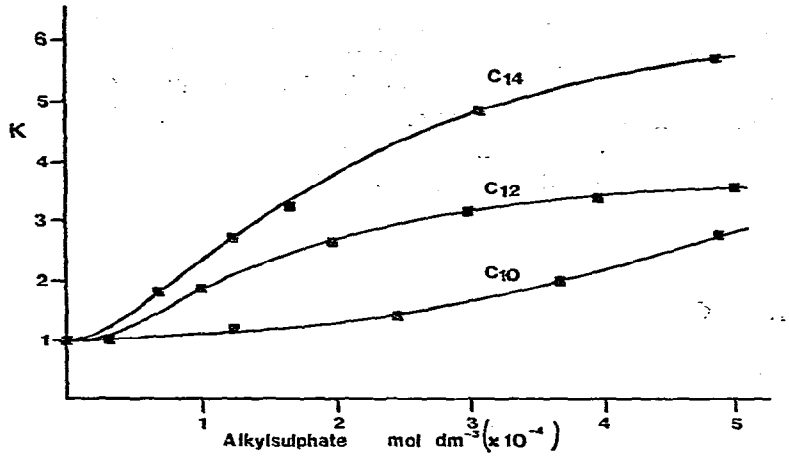

Fig. 7. Relationship between capacity ratios of tryptophan and mobile phase concentrations of an homologous series of long-chain alkylsulphates at pH 2.1. Stationary support, Spherisorb ODS (5 μm) eluent, methanol–water (1:1); the homologue number is indicated next to each data line.

solutes has recently led us to develop a HPLC method which has octadecyl reversed-phases, ABDACs as pairing ions and aqueous methanol as eluent. In two papers[74,78] the efficiency of these pairing ions is demonstrated by the chromatographic performance of the anti-allergy drug sodium cromoglycate in both simple aqueous solutions and in urine. In addition, the mechanisms of retention have been further investigated by reference to the behaviour of a series of acid red dyes in ABDAC columns. Fig. 8 shows how the capacity ratio of sodium cromoglycate alters with increase in ABDAC concentration (0–6 $\times$ 10^{-4} mol·dm^{-3}) for different ABDAC homologues. By study-

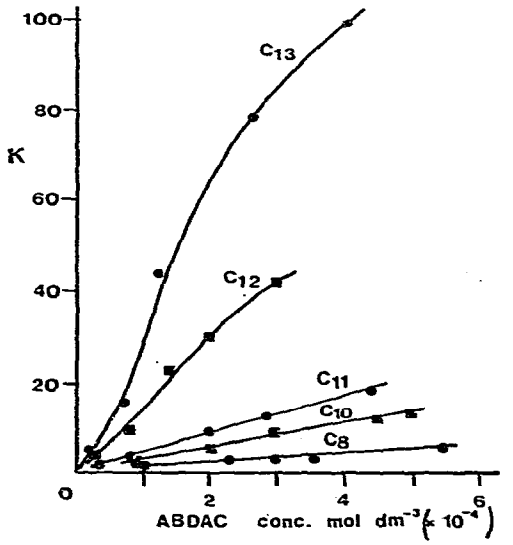

Fig. 8. Relationship between sodium cromoglycate capacity ratios and ABDAC mobile phase concentrations for a number of ABDAC homologues. Conditions as for Fig. 7.

ing column breakthrough times for the ABDACs we have been able to show that for the C_8 to C_{13} ABDAC homologues there is a linear relationship (*cf.* Knox and Laird[70]) between uptake by the stationary support and ABDAC mobile phase concentration. Evidence presented showing bulk-phase extraction of the acid red dyes as ABDAC ion pairs and other indirect evidence has caused us to suggest the following scheme to explain retention in surface-active ion-pair HPLC:

$$\begin{array}{ccc} A_m^+ + B_m^- & \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} & (A,B)_m \\ K_{-1}\Big\| K_1 & & K_{-4}\Big\| K_4 \\ A_s^+ + B_m^- & \underset{K_{-5}}{\overset{K_5}{\rightleftharpoons}} & (A,B)_s \end{array}$$

Scheme 4.

where A^+ is the ABDAC counter ion; the subscripts s and m refer to stationary and mobile phase: $K_1 = [A^+]_s \cdot [A^+]_m^{-1}$; $K_2 = [A,B]_m \cdot ([A^+]_m \cdot [B^-]_m)^{-1}$; $K_4 = [A,B]_s \cdot [A,B]_m$ and $K_5 = [A,B]_s \cdot ([A^+]_s \cdot [B^-]_m)^{-1}$. Two prime overlapping retention mechanisms (which would explain the observed sigmoidal capacity ratio *versus* ABDAC concentration) are suggested. That is, ion-pair formation in the mobile phase of the water-structure enforced[9] type, followed by distribution to the stationary phase (Scheme 4, K_2 and K_4), and ion-pair formation with the adsorbed pairing ion via an ion-exchange process (Scheme 4, K_5). The extreme flexibility of the ABDAC system afforded by alteration in either ABDAC concentration and/or chain length is exemplified by the analysis of drugs in urine in which urine containing drug is directly injected to a system in which the ABDAC concentration is designed to elute the solute peak well away from the biological components' peak.

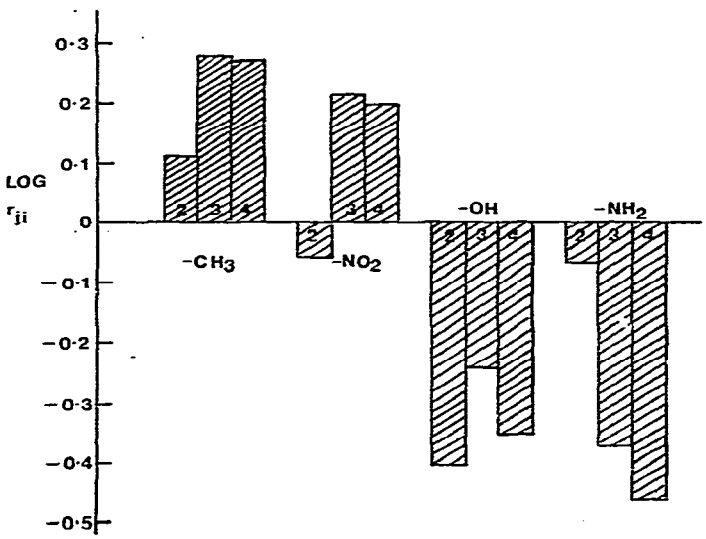

Fig. 9. Logarithms of selectivity factors (r_{ji}) for functional groups monosubstituted into the aromatic ring of benzoic acids determined in a C_{11}BDAC ion-pair HPLC system at 25°. C_{11}BDAC concentration $2 \cdot 10^{-3}$ mol·dm^{-3}; stationary support, Spherisorb ODS (5 μm); eluent, methanol–water (1:1).

Fig. 9 is an attempt at rationalizing the behaviour of functional groups[79] in ABDAC ion-pair systems by presenting r_{jl} (selectivity factor) values (eqn. 25) for various substituent groups.

Recently Kraak *et al.*[73] have shown that the addition of small amounts of anionic surface active agents (sodium dodecylsulphate, sodium dodecylsulphonate and dinonyl naphthalenesulphonic acid —*cf.* section IV.1.d) to the mobile phase of a reversed-phase HPLC system caused such systems to behave like conventional ion-exchange systems. For a series of amino acids the degree and order of retention was influenced by temperature, the hydrophobic support, the pH and the nature and concentration of the anionic surfactant, organic modifier and surfactant counter ion in the eluent. Unlike others[70,78], these workers argue that since the amount of surfactant adsorbed is determined by its adsorption isotherm, and since this may not be easily obtained, reference to this amount brings a high degree of uncertainty into the discussion of solute retention. Hence they derive an expression which describes the dependence of the total distribution coefficient of a sample on the pH and the surfactant's counter-ion concentration on one hand and the two pK values of the amino acid on the other. Although most of the changes in constitutional and environmental parameters produced similar effects to those outlined for other studies, the effects of surfactant counter-ion concentration and temperature merit further attention. Kraak *et al.*'s model assumes that if the pH, surfactant concentration and organic phase modifier concentration are kept constant, then a linear relationship must exist between the reciprocal of the capacity ratio and the counter-ion concentration. And indeed this is the case as shown by Fig. 10 which is taken from ref. 73. This result is taken to prove that at high surfactant concentrations (0.5%, w/v) ion exchange is the prime retention mechanism, and also shows that the counter-ion concentration is another variable which may be altered to increase sample separation and elution.

Kraak *et al.* choose to examine rather high surfactant concentrations (0.1–1.0%, w/v) in their study, and for the temperature effect a 0.3% sodium dodecyl-

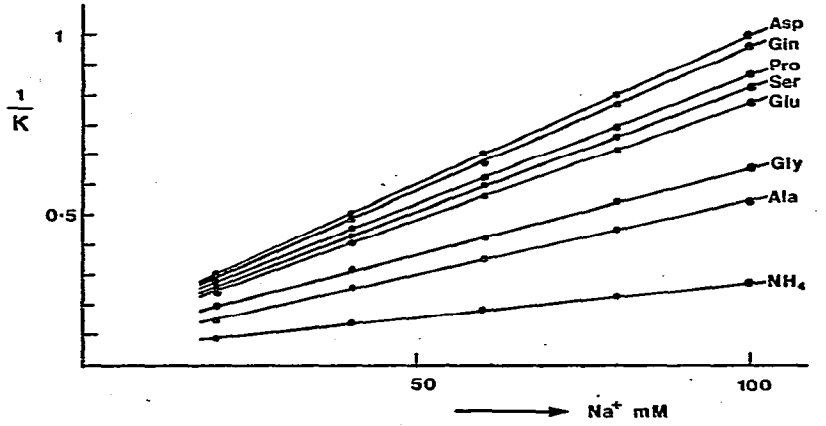

Fig. 10. Dependence of the reciprocal of the capacity ratio of amino acids on the pairing ion's counter ion (Na^+) concentration in the mobile phase. Support, RP-8; eluent, 0.01 mol·dm^{-3} citric acid (pH 2.25)–1-propanol (9:1)–Na_2SO_4 (0–0.08 mol·dm^{-3}); pairing ion, sodium dodecylsulphate, 0.5% (w/v); temperature, 25°. This figure is Fig. 6 of ref. 73.

sulphate concentration was studied over a 20–50° range. For all experiments the amino acid capacity ratios decreased with temperature, and in some cases the order of elution changed considerably. Although an increase in temperature will cause a decrease in hydrophobic interactions due to a loss in water structure, these results have been explained[73] on the basis that the effect is caused mainly by changes in the ion-exchange equilibrium, rather than by a decrease in the amount of surfactant adsorbed. Perhaps more attention should given to this latter effect. Temperature will, of course, also affect the micellization–aggregation abilities of the surfactant and this could explain some of the results.

V. SEPARATING EFFICIENCY

The value of any technique is reflected in its ability to perform tasks more comprehensively, and perhaps more efficiently, than established methods. This contribution has shown in some detail how the various ion-pair HPLC techniques can help in the analysis of a variety of solutes. It now remains to be demonstrated how efficient such techniques can be.

The separating efficiency of a column is usually expressed either by the height equivalent to a theoretical plate, H, or by the reduced plate height, h. The variables which can affect H include column temperature, pressure, flow-rate, phase volume ratio, sample elution time, mobile phase viscosity etc. With ion-pair systems additional parameters include sample concentration and pairing-ion type and concentration. To simplify discussion on the relative merits of different ion-pair systems Table 4 has been constructed and attempts to relate H values to the different modes of ion-pair HPLC under approximately similar conditions of flow-rate and sample elution time; obviously the data presented are for experimentally optimised conditions. From this table it can be seen that there is a spread in optimal H values depending on both the nature of the pairing ion and the stationary support. Undoubtedly the better non-compressible supports of lower mean particle diameter give best column efficiencies. Perchlorate columns give good efficiency compared to other similar pairing ions, but not until the surface-active pairing ions are used does the efficiency approach that of straight adsorption chromatographic methods.

Retention times will affect H values depending on the nature of the support material. Using picrate as pairing ion it has been demonstrated[19] that when cellulose is the support it reaches a maximum when the sample capacity ratio approximates to 1, and decreases as the capacity ratio increases further. This indicates that stationary phase mass transfer and/or extraction kinetics dominate in the chromatographic behaviour. With DiaChrom as support better efficiencies can be demonstrated due probably to the lower mean particle size diameter; also the variation of H with capacity ratio is less pronounced than with cellulose, indicating that H is now dominated by combined Eddy diffusion and mass transfer effects[19].

Other factors which specifically relate to ion-pair HPLC and which can affect H are the nature and concentration of the pairing ion. It is interesting to note that for the naphthalene-2-sulphonate system it is believed[28] that the rate of extraction of the naphthalene-2-sulphonate–sample ion pair is critical in determining H.

The concentration of pairing ion relates to the column load buffer capacity (eqns. 14–18). Eksborg and Schill[19] have shown that with a straight-phase system using 0.06 mol·dm^{-3} picrate as pairing ion a sample loading up to $10^{-7.4}$ mol could

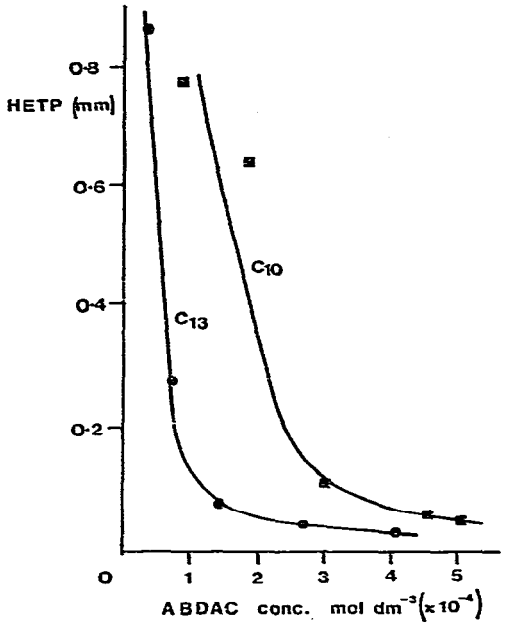

Fig. 11. Effect of ABDAC mobile phase concentration and chain length on the height equivalent to a theoretical plate using sodium cromoglycate as sample. The homologue number is next to each data line.

be achieved without an alteration in H (of 0.3 mm). However a loading of 10^{-7} mol led to an increase in H to 0.4 mm. Similarly with a picrate concentration of 0.014 mol·dm^{-3}, a 10^{-10} mol sample loading gave an H value of 0.5 mm which steadily rose to 1.3 mm as the loading was increased to *ca.* 10^{-7} mol. Our results[74,78] with the surfactant ABDACs as pairing ions in reversed-phase systems show that column efficiency is dependent upon both the nature (*i.e.* homologue number) of the pairing ion and its concentration (Fig. 11).

VI. FUTURE PERSPECTIVES AND CONCLUDING REMARKS

Ion-pair techniques undoubtedly have a place in the HPLC armoury for tackling normally difficult analyses of ionised solutes (such as drugs and their metabolites) in a variety of environments. Many uses of ion-pair HPLC for the direct analysis of solutes in biological fluids have been reported, some without the pre-extraction step (although the use of short pre-columns may be indicated[66]), and it would appear that these areas, *i.e.* of detection in formulations and biological fluids, will be those which gain most from use of ion-pair HPLC.

Additionally it should be possible to use or adapt the methods to perform enantiomer separations. Selective enantiomer extraction into bulk phases is now possible using adduct forming agents and it will surely not be too long before these techniques are successfully extrapolated to HPLC. Of great interest also are the recently reported studies of Mikeš *et al.*[89,90,93] in which the resolution of optical helicenes

TABLE 4

COLUMN EFFICIENCY IN ION-PAIR HPLC SYSTEMS

TPrA = Tetrapropylammonium; TBA = tetrabutylammonium; CTAB = cetyltrimethylammonium bromide; NS = naphthalene-2-sulphonate; ABDAC = alkylbenzyldimethylammonium chloride.

Phase system	*Support*		*H (mm)*	*Flow-rate (mm/sec)*	*Capacity ratio*	*Reference*
TPrA/chloroform–1-pentanol	Diachrom		0.37	2.0	2.1	55
TBA 0.03 *M*/1-pentanol	LiChrosorb	30 μm	0.8	1.7	5	27
	SI 60	10 μm	0.15	3.0	5	27
		5 μm	0.08	1.7	5	27
TBA 0.01 *M*/1-pentanol	LiChrosorb SI 60	30 μm	0.5	1.2	5	27
$TBAHSO_4$/1-butanol–*n*-hexane	LiChrospher SI 100	10 μm	0.05	2.0	5.4	32
			0.14	2.0	0.7	32
Picrate 0.06 *M*/chloroform–1-pentanol	Cellulose	30–65 μm	0.4	2.0	5	53, 19
	Porasil-D	37–74 μm	1.1	0.7	10	19
Perchlorate/cyclohexane–pentanol	Cellulose	30–65 μm	0.8	2.0	6.5	35
Perchlorate/butanol–methylene chloride	Silica gel	10 μm	0.07	5.0	6.3	25
NS 0.01 *M*/chloroform–pentanol	Lichrospher SI 100	10 μm	0.06	2.5	0.6	56
			0.23	2.5	4.4	56
Chloride/pentanol–water	Cellulose	30–65 μm	0.8	—*	—*	63
Bromide/pentanol–water	Cellulose	30–65 μm	0.8	—*	—*	63
CTAB 1%/propanol–water	SAS silica	7 μm	0.032	—	3.0	70
ABDAC 10^{-4} *M*/methanol–water	ODS	5 μm	0.040	—	5.0	78

* V_r = 40 ml.

is performed using microporous silica particles which have been physically coated with agents having appropriate chiral centres. Additionally the combined use[94] of metal ions, hydrophobic chelating agents and chemically bonded *n*-alkyl stationary reversed phases for resolving enantiomers shows much merit. Also of interest to us[91] is the use of ion-pair techniques to provide physicochemical parameters for ionised solutes for use in quantitative drug structure–activity relationships, and this application is currently being investigated.

To us ion-pair methods and their approach are logical ones and as such it should be possible (sections III and IV.4) to predict rationally optimum phase systems and solute retention behaviour. The methods are flexible with approximately nine prime parameters (*i.e.* nature and concentration of pairing ion, support, mobile phase, stationary phase, temperature, pH, ionic strength and counter-ion concentration) being adjustable to produce optimal conditions. It has been demonstrated that small changes in these parameters can affect separations, and by combining these changes with the high performances resulting from modern column technology very rapid and highly selective separations will result. Additional flexibility can be afforded by the method by making use of the fact that the solute elutes from the column as the ion pair and so (with for example straight-phase systems) sensitivity can be controlled (sections IV.1.b and IV.1.d).

Both the theoretical and experimental aspects of reversed phase ion-pair HPLC (*i.e.* in which the pairing ion is located in the eluent) suggest that this is the preferred method for using ion-pair techniques. This mode has the prime advantages that both the type and the concentration of the pairing ion can be easily changed even during an analysis, that gradient elution can therefore be carried out and that large injection volumes are possible. This approach does not suffer from column instability due to bleeding as occurs with straight-phase methods and nor are peak assymetries so apparent.

By far the most preferred approach would appear to us to be that employing surface-active pairing ions with chemically bonded stationary supports. Table 4 clearly shows how efficient such systems are compared to both other ion-pair methods and to non ion-pair HPLC techniques. The approach also gives rise to remarkably stable systems which have demonstrable flexibility in their use, as afforded by the possible two-dimensional change in their hydrophobicity and concentration. Surface-active agents used as pairing ions can generally be obtained in the pure state and have the advantage of being ionised under normal chromatographic pH values.

Because of these arguments and the previous discussions we suggest that the use of surface active ions in ion-pair HPLC will be the most profitable one for the chromatographic analysis of ionised molecules, especially when these molecules are situated in complex environments such as drug formulations and biological fluids.

Finally it is pertinent to echo the recent comment by Kissinger[92] that the method in all its modes should "not be viewed as being a well-understood extension of a very classical idea" (*i.e.* that of ion-pair partition); our own studies and those of others have definitely demonstrated an adsorption mode for surface-active pairing ions as well as ion pairing in the mobile phase. What needs to be done is to maximise and utilise these phenomena to produce even better and more sophisticated separations, and it is suggested that this can best be performed by first fully elucidating the mechanisms of retention in these systems.

VII. ACKNOWLEDGEMENTS

We are grateful to S. S. Davis, T. Higuchi, J. C. Kraak, H. Poppe and G. Schill for their discussions on ion-pair systems; to the Science Research Council and Imperial Chemical Industries for a postgraduate studentship for one of us (C.M.R.); and to Miss B. C. Lester for her valued secretarial assistance.

VIII. SUMMARY

The analysis of ionized solutes by high-performance liquid chromatography (HPLC) may be facilitated by the use of ion-pairing agents. This contribution discusses the development of ion-pair HPLC during the past decade, and points to those aspects of the technique which have greatest significance. Mathematical descriptions of various probable ion-pair retention mechanisms are given and related to the practical usage and efficiency of the methods. A comprehensive listing of reported chromatographic systems, pairing ions and solutes studied is given.

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