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Review

Ion-pair extraction and high-performance liquid chromatography in pharmaceutical and biomedical analysis

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Introduction

Pharmaceutical and biomedical analysis is increasingly demanding in terms of selectivity and sensitivity. Satisfactory analytical strategies in these areas are of vital importance to drug development and handling, and often have demands placed upon them that may arise from regulatory sources. The present contribution aims to describe how the use of ion-pair formation and distribution can assist in meeting these demands, by utilizing the techniques of liquid–liquid extraction and high-performance liquid chromatography (HPLC).

Ion-pair Extraction

The analysis of nonelectrolytes having suitable spectral properties is generally relatively simple. However, many compounds of pharmaceutical and biomedical interest, including drugs, formulation additives, phytochemicals and endogeneous materials such as peptides and vitamins, are ionized or ionizable. To remove unwanted chemicals from the body, metabolic processes generally yield metabolites that are more hydrophilic and conjugated with water-soluble acids. By their very nature, these polar ionized compounds are difficult to isolate for subsequent assay, particularly when they are found in biological fluids such as bile, plasma and urine. Although for weak acids and bases it is often possible to adjust the pH to give the unionized, extractable form, for those compounds with pK_a values outside the range 3–9 the drastic extraction conditions needed can lead to problems of solute stability. Additionally, for aprotic solutes such as quaternary ammonium compounds, and materials which are ionized at all pH values

(e.g. sulphonic acids, amino acids, aminophenols and metabolite conjugates), pH adjustment techniques are not possible.

However, it has been established for over 40 years that, although the primary equilibrium may involve the formation of an ionized solute, the physicochemical nature of this ionized species can be altered by introducing a second equilibrium, leading to the formation of ion-pairs. Ion-pairs possess a low aqueous solubility and their prime property in an analytical context is their tendency to move from areas of high polarity, e.g. water, to areas of low polarity, such as organic solvents. Since ion-pair formation can be highly specific, and since transfer to the organic phase can involve not only hydrophobic forces but polar–polar and specific-solvent interactions, then the combination of ion-pair formation and distribution can afford great selectivity to a given extraction procedure.

Design of an extraction system

Some years ago Jonkman reviewed [1, 2] the technique of ion-pair extraction as an isolation technique in pharmacy for the extraction of drugs from biological fluids. He concludes that if environmental and constitutional factors are well chosen, very 'pure' extraction can be possible, with nanogram amounts of material being detectable. Interestingly, Jonkman observes that even in 1931 strychnine was isolated from Easton's syrup using an ion-pair technique. In this method, as with all methods up to the 1960s, the pairing ions used were mostly inorganic or polar organic ions, involved in extractions which at best could be described as empirical. With such pairing ions, the extractions of a weak acid HA in the presence of a pairing ion Q^+ can be expressed as:

$$HA \leftrightarrow H^+ + A^- \overset{Q^+}{\leftrightarrow} (A,Q)_{int} \leftrightarrow (A,Q)_o$$

Scheme 1

where $(A,Q)_{int}$ indicates the ion-pair formed in the interface layers between the aqueous and organic solvent phases; and $(A,Q)_o$ indicates the ion-pair in the organic phase. The extraction constant, K_{EX}, is given by:

$$K_{EX(A,O)} = \frac{[A,Q]_o}{[A^-]_{aq} \cdot [Q^+]_{aq}} \quad (1)$$

where $[A^-]_{aq}$ and $[Q+]_{aq}$ represent the concentrations of anion and pairing ion in the aqueous phase. Although this, and following equations, can be modified to take account of ion-pair formation in the aqueous phase [3], it is convenient to discuss extraction in terms of Scheme I, with the assumption that the concentration of ion-pair in the aqueous phase tends to zero, and that hydrophobic effects can modify such ion-pairing. With no transfer of the pairing ion, the distribution ratio, D_A, for the formed ion-pair is:

$$D_A = \frac{[A,Q]_o + [A^-]_o}{[A^-]_{aq}} = K_{EX(A,O)} \cdot [Q^+]_{aq} \cdot \left(1 + \frac{K_{diss}}{[Q^+]_o}\right) \quad (2)$$

where the dissociation of the ion pair in the organic phase, K_{diss}, is given by:

$$K_{diss} = ([A^-]_o \cdot [Q^+]_o)/[A,Q]_o. \quad (3)$$

K_{diss} will be of significance only when the extracting phase has a sufficient polarity. Generally, since ion-pairs formed with many ions of opposite charge have some polarity, the more polar the extracting phase the higher will be the extraction of the ion-pair. This simplification ignores the possibility of specific solvation interactions occurring in the organic phase, a point to be returned to later. From equations (1) and (2), and invoking simple Nernstian distribution behaviour, it can be shown that the fraction of solute, f, transferring to the organic phase as the ion-pair in a single extraction sequence is given by:

$$f = (1 + V_{aq}/V_o \cdot D)^{-1} \quad (4)$$

where V_{aq} and V_o are the volumes of the aqueous and organic phases respectively. From Scheme (**1**) and equations (1)–(4) it follows that a number of factors will affect ion-pair extraction, as summarized in Table 1.

Table 1
Factors affecting ion-pair liquid–liquid extraction

Pairing ion	size hydrophobicity concentration
Extracting phase	polarity specific solvation presence of adduct-forming agents volume
Aqueous phase	salt concentration pH volume
Nature of the ion pair	size hydrophobicity specific solvation behaviour dissociation constant
Temperature	

Nature of the extracting phase

The significance of the extracting phase can be seen from Table 2, which gives the logarithmic extraction constants for tetraalkylammonium–picrate ion pairs. It is clearly demonstrated that the dielectric constant of the organic phase (which reflects solvent polarity) can indicate the relative extractive ability of solvents. This can be explained by supposing that the more polar the organic solvent, the greater will be the solvation of the slightly polar ion-pair. Moreover, the greater the solvent polarity, then the greater will be the possibility for ion-pair dissociation in the organic phase (equations 2 and 3). For example, Jonkman has described the extractive ability of halogenated hydrocarbons for a thiazinium–perchlorate ion pair as: dichloroethane > dichloromethane > chloroform.

Table 2
Extraction constants of tetraalkylammonium-picrate ion pairs

Cation*	Extracting phase	Dielectric constant	Log extraction constant	Reference
TEA	Methylene chloride	8.9	2.34	4
TBA			6.68	4
TEA	Chloroform	4.8	1.32	5
TBA			5.91	5
TBA	Benzene	2.3	3.59	5
TBA	Carbon tetrachloride	2.2	5.91	5
TMA	Chloroform–pentan-1-ol (19:1 v/v)		−0.51	6
TEA			1.46	6
TBA			6.60	6

* TMA, TEA and TBA are tetramethyl-, tetraethyl- and tetrabutylammonium.

This mirrors the capability of these ion-pairs to dissociate in these solvents. As well as solvent polarity and dissociation, consideration should also be given to the possibility for specific solvation of the formed ion-pair in the organic phase. Higuchi *et al.* [7] have considered the mechanism of ion-pair extraction. For dextromethorphan–hydrobromide ion-pairs, they found that the extractive ability of various solvents is chloroform > nitrobenzene > cyclohexanone, although on the basis of solvent polarity the order nitrobenzene > cyclohexanone > chloroform would be expected. Their results clearly demonstrate that the specific hydrogen-bonding interaction between the acidic protons of chloroform and the hydrobromide, which of the two ions will have the highest residual charge density, is more favoured than the nitrobenzene (proton acceptor)–dextromethorphan interaction.

In general, however, the overall effect of ion-pair distribution is given more consideration in extraction design than are the subtle, though significant, specific solvation processes. The use of halogenated hydrocarbons and aliphatic alcohols of low chain length (e.g. pentanol) usually give distribution ratios which ensure more than 99% extraction of both hydrophobic and moderately hydrophilic ion-pairs.

For the extraction of hydrophilic ion-pairs that are formed between ions having small molecular weights or polar groups, extraction can be modified by the incorporation of adduct-forming species in the extracting phase. For example, the extraction of phenoxymethylpenicillin into halogenated hydrocarbons can be greatly enhanced both by adjustment of pH (Table 1) and by the addition of dibenzo-18-crown-6 to the organic phase [8]. Since pure crown ethers are commercially available, this method may provide a highly selective means of extracting polar ions. More examples of the use of adduct formation will be given later.

Nature and concentration of the pairing ion

From equation (2) it may be seen that both the nature and the concentration of the pairing ion will influence extraction. It is usually found that for pairing ions which do not self-associate or form polymers with the solute ion, an increase in their concentration leads to an increase in extraction. The nature of the pairing ion generally affects extraction in two ways: firstly, the more hydrophobic the pairing ion, then the greater will be the ion-pair formation constant; and secondly, the larger the pairing ion, then the greater will be the transfer of the formed ion-pair into the organic phase. Tables 3 and 4

Table 3
Effect of pairing ion type on extraction of tetrabutylammonium ions between water and chloroform [9]

Pairing ion		Log extraction constant*
Inorganic ions	hydroxide	−1.67
	chloride	−0.11
	bromide	1.29
	nitrate	1.39
	iodide	3.01
	perchlorate	3.48
Organic ions	phenate	0.05
	acetate	−1.21
	phenylacetate	0.27
	benzoate	0.39
	naphthalenesulphonate	3.45
	trinitrobenzenesulphonate	4.47
	picrate	5.91
	methylorange	5.47

* These are conditional extraction constants which account for side-reactions, etc.

Table 4
Extraction of organic cations from water to chloroform using radiolabelled Rose Bengal (from [10])

Solute ion	Molecular weight	Extraction relative to MIP
Monoquaternary ammonium ions		
Tetraethylammonium	136	0
3-Methyl-3-phenylimidazole (1,2a)-pyridinium (MIP)	210	1
Neostigmine	223	0
Dibenzyldimethylammonium	226	322
Hexadecyltrimethylammonium	285	1216
Tribenzylmethylammonium	302	288
Bisquaternary ammonium ions		
Succinylcholine	290	0
Paraquat	186	376
Fazadinium	444	3263
Pancuronium	572	671
(+)-Tubocurarine	625	617

illustrate these principles quite clearly. The effect of pairing-ion concentration and hydrophobicity is demonstrated by Fig. 1, which shows the distribution of the antiallergic drug sodium cromoglycate between water and chloroform as ion-pairs with some quaternary ammonium hydrophobic ions [11]. Table 5 gives the more commonly used pairing ions. Although the action of some of these could be described as being via 'salting-out', equations (1) and (2) indicate that generally, the greater the concentration of pairing ion, the greater will be the extraction of the solute ion.

Much of the work on the use of adduct formation for enhancing ion-pair extraction is due to Schröder-Nielsen, who has examined a large variety of adducts. Of particular

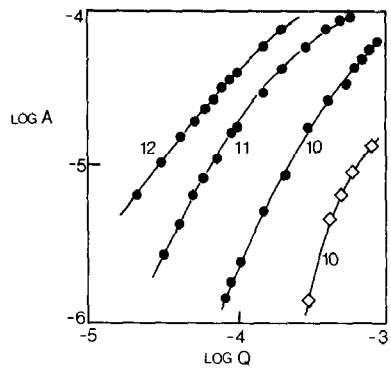


Figure 1
Amount of sodium cromoglycate A ($M \times 10^4$), extracted as the 2:1 ion-pair with a series of homologues of alkylbenzyldimethylammonium chlorides, Q, (homologue number, given next to each datum line) at various concentrations (M). Extraction is between water and chloroform, with an initial sodium cromoglycate concentration of 1×10^{-4}M. Diamonds represent extraction in the presence of 0.01 M NaCl (Ref. 11).

pharmaceutical interest is this worker's study [12] on the extraction of salicylate and other anions as ion-pairs using hydrophobic alkylammonium ions, and on the effect of trioctylphosphine oxide as adduct-forming agent in such extractions. This study considered the use of very hydrophobic pairing ions to enhance polar ion extraction, although limited by the decreasing aqueous solubility of these pairing ions with increasing hydrophobicity. Adduct-forming agents increase the capacity of the organic phase to accommodate the formed ion-pair, and hence increase the extraction potential. Schröder-Nielsen was able to demonstrate in a dramatic way that, although non-polar solvents such as hexane had little affinity for formed ion-pairs, the presence of an adduct-forming agent at a concentration of 1×10^{-2}M led to a marked increase in the extent of ion-pair extraction. It has been found also that dibenzo-18-crown-6 has a strong preference to form adducts with primary ammonium ion pairs [13]. This suggestion of a stereo-specific bonding of the crown ether to the primary ammonium of the ion-pair further illustrates the specificity of extraction possible with ion-pair extraction techniques.

Table 5
Pairing ions commonly used in liquid–liquid ion-pair extraction

Anions
Bromothymol blue
Picrate
Trichloroacetate
Hexa- to dodecylsulphate
Perchlorate
Rose Bengal
Cations
Tri- and tetraalkyl ammonium (methyl to butyl)
Hexadecyltrimethylammonium
Alkylbenzyldimethylammonium (octyl to tetradecyl)

Salt, pH and temperature effects

Table 1 indicates the other environmental factors which play a role in ion-pair extraction. Of particular practical importance are the effects of salt and pH. Temperature effects are almost totally disregarded in extraction techniques, although an

increase in temperature is likely to cause a slight decrease in the extraction coefficient. Equation 2 and Scheme (**1**) indicate that the greater the degree of ionization of the solute and pairing ions, then the greater will be the extraction coefficient. To avoid problems of partial ionization, it is advisable to adjust the pH of the aqueous phase so that both ions are at least 99% ionized, although later in the discussion on HPLC, it will be seen that selectivity manipulation can be effected through pH control.

By contrast with conventional (non-ion-pair) extractions, the addition of salt (NaCl, etc.) tends greatly to decrease the extraction of ions as ion-pairs. This may be due to a number of factors. For the example given in Fig. 1 the reduced extraction of cromoglycate ion may be attributable to a reduction in the rate of initial ion-pair formation between it and the quaternary ammonium ion. For other extracting systems the decreasing extraction coefficient of the prime ion pair may result from competition by the added salt ion. This may in practice result in an increased degree of extraction of the prime cation, which may or may not be desirable. Thus, it is advisable to reduce the amount of added salt in an extraction system to as low a level as possible (ignoring the pairing ion counter-ion).

Jonkman has described the extraction of almost 90 drugs as ions by ion-pair extraction techniques. Although it is not the intention to update that review, of particular pharmaceutical interest is the more recent work by Fransson and Schill [14] on the use of small alkylammonium ions for the ion-pair extraction of glucuronic, sulphuric and glycine conjugates of benzoic and cholic acid derivatives. The study by Hurwitz and Carney [15] on the improvement of the partition behaviour of ampicillin using various quaternary ammonium compounds is also of interest.

Enhancement of detection

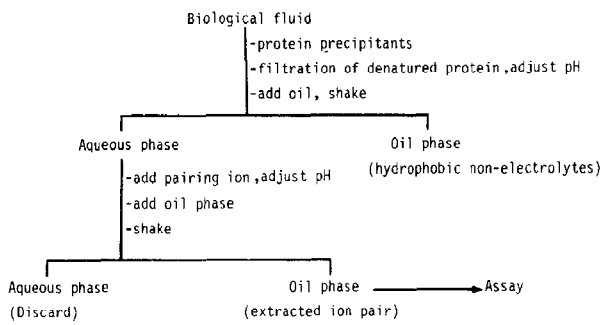
As well as being used to effect ion extraction, pairing ions may be chosen in order to improve the detection of the solute ion. For example, from Table 5 it is seen that bromothymol blue is commonly used as a pairing ion. Clearly an ion with such a high extinction coefficient would be an ion of choice for the sensitive assay of compounds with a low extinction coefficient. In a similar manner the concept of employing pairing ions that confer high fluorescence on non-fluorescent solute ions (as the ion pair) is attractive. Borg [16] suggested the use of dimethylprotriptylinium for the extraction and simultaneous detection of inorganic anions and organic sulphonates. Westerlund and Borg [17] have proposed the use of anthracene sulphonates for the assay of amines and quaternary ammonium ions. Similarly, Dent *et al.* [18] have shown that sodium coumarin-6-sulphonate at a concentration of 1×10^{-2}M can be used at pH 5 in the assay of tertiary amines such as chlorpheniramine maleate, at concentrations of the free base greater than 35 ng/ml. It will be shown later that these concepts are of practical use in HPLC analysis.

Sample handling

The complex nature of biological fluids requires that samples often need to be subjected to a pre-treatment step before extraction of the required solute. For ion-pair extractions it has been found in the author's laboratories that the protocol outlined in Scheme (**2**) will generally give satisfactory results. After a possible protein precipitation step, unwanted hydrophobic materials are extracted by the organic phase; this may follow adjustment of pH so that materials to be extracted are in the unionized form. After phase separation, and possible further pH adjustment, an appropriate pairing ion

is added to the protein-free filtrate and the extraction repeated, so that the desired ion-pairs are selectively extracted. If HPLC techniques are to be used, then quite frequently direct injection of the initial protein-free filtrate can be made.

Although simple shake-flask methods are used for extractions, continuous segmented-flow techniques can be used for materials that require no pretreatment step. For example, Karlberg *et al.* [19] and Kinkel and Tomlinson [20] have shown how ion-pair extractions can be followed by injecting samples into continuously moving streams of segmented organic and water phases. With on-line phase-splitting and detection, these methods permit upwards of 80 samples per hour to be analysed. Coupled with pre-treatment steps and on-line injection into chromatographic systems, such techniques will help in greatly increasing the selectivity possible with ion-pair extraction methods.



Scheme II
Treatment of biological sample for ion-pair extraction.

Ion-pair High-performance Liquid Chromatography

Liquid–liquid extraction techniques are of importance in sample treatment prior to the assay step. However, to achieve high selectivities and sensitivities recourse has to be made to modern liquid chromatographic techniques. Although ion-pair effects have been utilized quite successfully in paper and thin-layer chromatography, it is only in high-performance liquid chromatography that the remarkably flexible nature of these effects can be fully exploited [21]. Indeed, a recent review of the literature [22] shows that there are over 400 references to the use of ion-pair effects in HPLC. This popularity reflects the experience of many who have found that the method can lead to efficient, selective assays, with the added advantage that improvement in sensitivity of solute ions is possible. The employment of these techniques arises also because ion-exchange methods are often inefficient, irreproducible and unstable, and because ion-suppression methods for weak acids and bases generally lead to very poor sample resolution.

Construction of the system

It is convenient to describe retention in terms of an extraction model equations (1–2), although dynamic ion-exchange [23] and mixed partition/adsorption [24] models can be invoked. Thus, for reversed-phase HPLC the capacity factor, k', of a weak acid anion, A^-, eluted as the ion-pair (A,Q) with a pairing ion Q^+ is given by:

$$k' = K_{EX(A,Q)} \cdot [Q^+]_m \qquad (5)$$

where the phase ratio is taken as unity. The extraction constant is given by:

$$K_{EX(A,Q)} = \frac{[A,Q]_s}{[A^-]_m \cdot [Q^+]_m}, \quad (6)$$

where the subscripts m and s refer to mobile and stationary phases respectively. Similarly, for a straight-phase system, retention is given by:

$$k' = (K_{EX(A,Q)} \cdot [Q^+])^{-1}. \quad (7)$$

Figure 2 shows how the construction of the phase system, the concentration of the pairing ion and the effect of differing extraction constants (brought about by *inter alia* changes in pairing ion type, phase composition, temperature and pH) can affect solute retention as the ion-pair. Careful construction of the chromatographic system can result in selective and efficient chromatography.

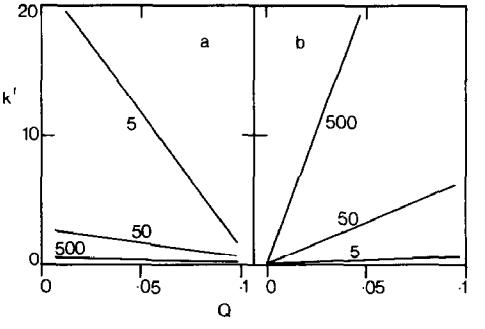


Figure 2
Relationship between solute capacity factor, k', and pairing ion concentration, Q, in: (a) straight-phase and (b) reversed-phase ion-pair HPLC systems. Numbers next to lines refer to different extraction constants (Ref. 24).

Liquid–liquid HPLC

The development of unique liquid–liquid HPLC systems for use with pairing ions of differing types has been due almost entirely to Schill and his group in Uppsala, although Kraak and Huber [25] were also prominent in the early literature with respect to demonstrating the applicability of specific solvating processes and competing equilibria in liquid–liquid straight-phase HPLC. The work of the Swedish group has been often reviewed, and the interested reader is directed towards reference [26]. Although a serious disadvantage of liquid–liquid systems, where the stationary phase is mechanically adsorbed onto the surface of a suitable packing material, is their instability and the limitations for gradient elution, their use is indicated for the detection enhancement of non-uv absorbing solute ions, as described below.

Variables affecting ion-pair HPLC

Nine principal parameters can be identified [21] as being readily adjustable to produce optimal solute selectivity and retention characteristics in ion-pair HPLC. For the reversed-phase mode these parameters and their effects are given in Table 6. Not included is the effect of the stationary support, which in reversed-phase systems has an effect depending upon the nature and amount of apolar characteristic [27]. Kirkland and Synder [28] have indicated that for ion-pair techniques the advantages of reversed-phase systems far outweigh those of straight-phase methods: the former are more stable, since

Table 6
Adjustable variables in reversed-phase ion-pair high-performance liquid chromatography

Variable	Effect	Comment
Nature of pairing ion	Generally the larger and more hydrophobic the pairing ion, the greater will be the retention and the selectivity	Alkyl chain variation is the easiest way to vary this
Concentration of pairing ion	Linear increase in retention with increase in pairing ion concentration for small pairing ions. For surface-active ions an increase in concentration will increase retention to a limit, beyond which further increase causes a fall; generally no effect on selectivity	For any system determine the maximum. The more hydrophobic the pairing ion the longer it takes for the column to reach equilibrium
Concentration of organic modifier	Retention and selectivity decrease with increase in concentration	A range of 10–70% modifier is used; a 10% increase in modifier can result in up to a three-fold decrease in capacity factor
Type of organic modifier	Less polar modifiers lead to a decrease in retention. Selectivity is highly dependent upon the type of modifier employed	A suggested eluotropic series is propanol > tetrahydrofuran > acetonitrile > methanol
Stationary phase	Greater retention with a more hydrophobic phase; some effects on polar solute selectivity	Use only monomeric bonded phases, since poor peak shape is observed with the polymeric type. With liquid–liquid systems the phases must be mutually presaturated; efficient temperature control is required
pH	As pH changes to make the solutes (and pairing ions) more ionized, retention will increase; possibility for alteration in phase selectivity	Operating range pH 1.5–7.5 with silica phases. The effect of buffers can be to disturb peak shape and to enter into competing additional equilibria
Temperature	Retention and selectivity generally decrease with increase in temperature	Although at higher temperatures better efficiencies can be achieved, it is best to work at lower temperatures
Ionic strength	Increase in ionic strength leads to a fall in retention, but no effect on selectivity	Probably due to disruption of ion-pair formation

the pairing ion is nominally in the polar phase and there is no bleed of pairing-ion; aqueous samples can be added directly to the system; less sample clean-up is necessary, since components of little interest in biological samples are usually polar and elute with the mobile phase front; solvent strength and polarity are readily adjustable (with bonded phases) using different amounts and types of pairing ions and organic modifiers; and selectivity can be altered by means of pH changes. In the following sections, therefore, the manipulation of the ion-pair effect in reversed-phase HPLC using chemically bonded solid stationary phases will be described.

1. *Nature and concentration of pairing ion.* From Scheme (**1**) and equation (1) it is clear that as ion-pair formation increases, there is an increase in the distribution ratio, which for reversed-phase systems leads to an increase in retention (equation 6), with the opposite effect (equation 7) for straight-phase systems. Since the physicochemical characteristics which determine ion-pair formation are charge, hydrophobicity and ionic radius, ion-pairs with high stability constants will generally have a high solubility in the organic phase. Table 7 lists those pairing ions in common usage. Not included are the inorganic ions, or those ions suggested for increasing solute ion detection. Except for perchlorate, all the pairing ions given are hydrophobic with molecular weights generally above 100; some can be classed as surface-active ions. The simplest method to alter pairing-ion hydrophobicity is through the alkyl chain-length. However, above an alkyl chain-length of seven the pairing ions become surface-active. These ions enter into other equilibria, including micelle formation and adsorption onto the surface of apolar stationary phases. With these pairing ions a nonlinear relationship between solute capacity factor and pairing-ion concentration is often found. The descriptions of solute retention given by equations (6) and (7) are inadequate to describe such relationships. Using phenomenological models,* retention can be described [29] for reversed-phase HPLC by:

$$k' = (k'_o + K_1K_3[Q^+]) \cdot (1 + K_1[Q^+])^{-1} \cdot (1 + K_2[Q^+])^{-1} \qquad (8)$$

where k'_o is the capacity factor of the ionized solute in the absence of pairing ion Q^+, and K_1, K_2 and K_3 refer to the ion-pair formation constant, the pairing-ion binding constant (with the stationary phase) and the ion-pair distribution constant respectively. This description expresses the parabolic dependency of k' on $[Q^+]$, provided that K_1^{-1} is greater than $(K_2[Q^+])^{-1}$. Results typically found are given in Fig. 3 [27], where it is seen that, although pairing-ion concentration can have a complex effect on solute ion retention, there is no effect on selectivity. It is suggested that it may be helpful during method development to identify the maxima and use pairing-ion concentrations at this point.

2. *pH.* For weak acids and bases it is frequently possible to control solute retention and selectivity by adjustment of the pH [27, 30] although it is common practice to ensure that

* Although the Horváth *et al.* model is able to describe the observed nonlinearity, it suffers from the disadvantage that electroneutrality conditions are not taken into consideration [3]. Although other models have been proposed, based on, *inter alia*, micellization behaviour, or combinations of a limited support adsorption capability with eventual associations in the mobile phase, their discussion lies outside the scope of the present review.

Table 7
Pairing ions commonly used in ion-pair HPLC

Anions	A	perchlorate
	B	picrate
	C	pentane sulphonate
	D	hexane sulphonate
	E	heptane sulphonate
	F	octane sulphonate
	G	octylsulphate
	H	dodecylsulphate
	I	9.10-dimethoxyanthracene sulphonate
Cations	J	tetramethylammonium
	K	tetraethylammonium
	L	tetrapropylammonium
	M	tetrabutylammonium
	N	dodecyltrimethylammonium
	O	tetradecyltrimethylammonium
	P	hexadecyltrimethylammonium
	Q	decylbenzyltrimethylammonium

Most of these pairing ions are commercially available from either Fisons Scientific Apparatus Ltd or Waters Associates Ltd.

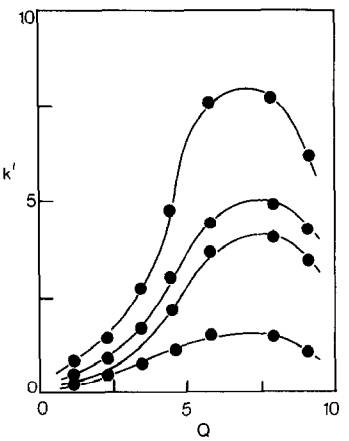


Figure 3
Effect of pairing-ion (Terdecylbenzyldimethyl-ammonium) concentration, Q, ($M \times 10^4$), on the retention of a series of substituted benzoic acids. Chromatographic conditions: Stationary phase, Spherisorb ODS; mobile phase, methanol–water (1:1 v/v); 0.025 M K_2HPO_4 (pH 7.5), at 30°C (Ref. 27).

all ions are in the fully ionized state. The effect of pH on the retention mode given by equation 8 can be expressed as:

$$K' = \frac{(k'_o + K_1^o K_a/[H^+] + K_1 K_3[Q^+])}{(1 + K_a/[H^+] + K_1[Q^+])\,(1 + K_2[Q^+])} \qquad (9)$$

where K_a, $[H^+]$ and K_1^o represent the acid dissociation constant, hydrogen ion concentration and the solute-stationary phase binding constant of the uncharged species respectively. Equation (9) predicts that the retention of a weak base will increase with decrease in pH, provided that the pairing-ion concentration remains constant. The opposite holds for weak acids. Figure 4 illustrates the effect of pH on the retention of an

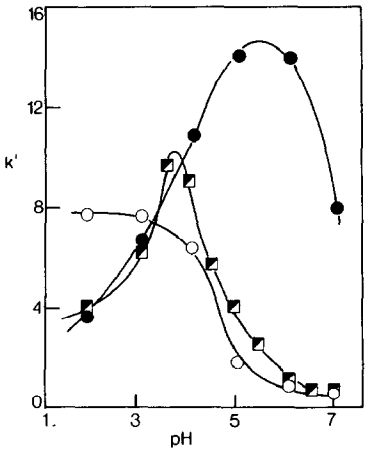


Figure 4
Effect of pH on the capacity factor, k', of *para*-aminobenzoic acid in the absence and presence of anionic or cationic pairing ions. Chromatographic conditions: Stationary phase, Spherisorb S5 ODS; mobile phase, methanol–water (1:9 v/v), 5×10^{-4}M pairing ion, 0.025 K_2HPO_4 (pH adjusted with H_3PO_4), 30°C. Key: open and closed circles represent dodecylsulphate and undecylbenzyldimethylammonium pairing ions respectively; squares indicate the absence of pairing ion (Ref. 30).

ampholyte, *p*-aminobenzoic acid, for which the pK_a and pK_b values are similar. In the absence of pairing ions, the k' vs pH relationship exhibits a sharp maximum at the isoelectric point. At low pH values, the ampholyte can pair with the anionic dodecylsulphate, and at higher pH values with the cationic quaternary ammonium salt. The example given in Fig. 4 demonstrates clearly that above pH 6 there is a dramatic fall in k', which has been attributed [30] to the presence of phosphate buffer components in the mobile phase. This pH range corresponds to the pK_a of phosphate for the change from dihydrogen- to monohydrogenphosphate. It appears that this latter phosphate species will associate more readily with the cationic pairing ion used than will the dihydrogen species, thereby reducing the effective concentration of the quaternary ammonium pairing ion. Selectivity is also modified by altering pH [30]. It has been suggested that this can be exploited to effect reversals in retention order of solutes with differing ionizable groups, as well as for structural isomers [30].

3. *Type and concentration of organic modifier.* The highly flexible approach obtained using different pairing ions at differing concentrations has meant that little systematic study has been carried out on the properties of mobile phase organic modifiers. Methanol and acetonitrile appear to be the modifiers of choice, the latter being apparently similar to methanol except for its ability to solvate ortho-substituted groups [31]. Tetrahydrofuran gives large alterations in system selectivity due to specific solvation effects. Its use as a modifier is suggested when hydrogen bonding effects need to be manipulated.

Since changing the amount of organic modifier in the mobile phase affects both retention and selectivity (Table 6), the combined effects of altering both pairing-ion concentration and modifier concentration provides an opportunity for further manipulation of chromatographic behaviour. This is exemplified by Fig. 5, which illustrates the effect that changing these two variables has on the retention of imidazole-4-acetic acid [30]. High concentrations of organic modifier tend to reduce the capacity ratios of solutes to such low values that, unless very efficient systems are employed, resolution and therefore selectivity can be lost. Thus it is often found that for ion-pair systems the range of modifier concentrations used lies between 10 and 70% v/v.

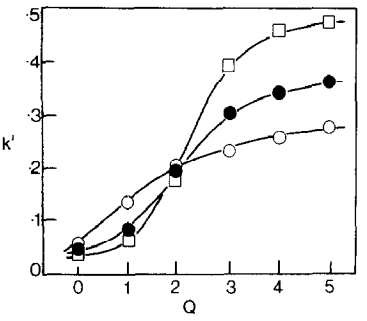


Figure 5
Effect of mobile phase organic modifier concentration and pairing ion, (Q), concentration (M) on the capacity factor of imidazole-4-acetic acid. Chromatographic conditions: stationary phase, Hypersil ODS; mobile phase, acetonitrile–water, 0 to 4 × 10^{-4}M tetradecylbenzyldimethylammonium chloride, 0.025 M K_wHPO_4 (pH 7.5), 30°C. Key: closed circles, squares and open circles represent 15%, 20% and 25% acetonitrile mobile phase concentrations (Ref. 30).

4. *Temperature, ionic strength and stationary phase effects.* Although the use of surface-active ions in reversed-phase HPLC can give columns with plate height values of less than 0.03 mm, it has been common practice with small alkyl ammonium ions to operate at 50°C to increase efficiency. An increase in temperature will, however, generally decrease both retention and phase selectivity [27].

From Fig. 1 it can be seen that the effect of inorganic salt is to reduce ion-pair distribution by disruption of ion-pair formation. This effect can be put to good use in ion-pair HPLC. Although the retention of solute ions as the ion-pair is decreased, the phase selectivity is not affected by the increase in ionic strength [27].

Octyl and octadecyl stationary phases have been the most widely used phases in ion-pair HPLC, although short alkyl side chain silica (SAS), bonded phenyl, DIOL and nitrile phases have also been reported. It has been argued that the length of bonded alkyl chains only affects the retention time. A recent study [27] has, however, demonstrated clearly that the effect of carbon loading on selectivity for polar groups is large. This probably reflects the influence of residual silanol groups, as much as the alteration in phase ratio.

Detection enhancement

Although gradient elution of the pairing ion can be used to concentrate components at the beginning of a column, in order to improve the overall method sensitivity, liquid–liquid ion-pair HPLC can permit a significant enhancement in solute detection. For straight-phase systems, since the sample ion is eluted from the column as the ion-pair, an ion with no uv absorption can be determined if its pairing ion, or the formed ion-pair, have suitable absorptivity.

For cationic solute ions, such as peptides and amino-acids, naphthalene-2-sulphonate has been proposed [26] for detection at the low nanogram level; for anionic solute ions, 1-(1-naphthylethyl)trimethylammonium [26] and *N,N*-dimethylprotriptyline [32] have been suggested. Mobile phases based on aqueous solutions of naphthalene-2-sulphonate and 1-phenethyl-2-picolinium using bonded-phenyl as stationary phase have recently been developed [33], to give more stable reversed-phase systems. In addition, sophisticated techniques have been developed recently by Frei and co-workers [34], who have investigated the potential of post-column extractor/detector systems for the determination of basic drugs, pesticides and their metabolites. Using dimethoxy-anthracene sulphonate, added either to the mobile phase or to the column effluent, and with post-column solvent segmentation by means of an extracting organic phase, these workers were able to show selective and sensitive analyses for such compounds. Undoubtedly these types of combined system have great potential in pharmaceutical and biomedical analysis.

Table 8
Recent applications of ion-pair HPLC in pharmaceutical analysis

Compound or class	Pairing ion*	Eluent†	Stationary phase	Ref
Acebutolol & metabolite	H	A (pH 3.5)	Micropak MCH 10	35
Allopurinol & oxypurinol	E	A (pH 5.9)	ODS	36
Amine drugs	I	B	Lichrosorb Diol	37
Aminoglycosides	C	C	ODS	38
Aminosalicylate/sulphapyridine	M,P	D	ODS	39
Amitriptyline	H	E	ODS	40
Anthraquinone glycosides	M	F	ODS	41
Apomorphine	H	G (pH 3.3)	Alkylphenyl	42
Atenolol	E	H	Nitrile	43
Barbiturates	J–L	I	Reverse phases	44
Bleomycins	E	F	ODS	45
Bromocriptine	E	F	ODS	46
Buflomedil HCl	H	I	ODS	47
Carbamazepine	K	J	ODS	48
Drugs of forensic interest	J–L	I	Reverse phases	44
Folic acid in multivitamin preparations	M	F	ODS	49
Gentamycin sulphate	E	I	ODS	50
Isoprenaline	H	I	ODS	51
Imipramine/desipramine & metabolites	E	K	ODS	52
Labetalol	P	L	ODS	53
Lidocaine/phenylephrine/betamethasone valerate	H	M	ODS	54
Moxalactam	M	M	ODS	55
Misonidazole & metabolites	E	F	ODS	56
Nalidixic acid & metabolites	P	M	Octyl	57
Oxyprenolol	F	F	ODS	58
Pentobarbital/pyrilamine	‡	N	ODS	59
Penicillins & cephalosporins	P	P	ODS	60
Phenothiazines	E	I	ODS	61
Pilocarpine	G	F	ODS	62
Polypeptide antibiotics	J	Q	ODS	63
Propranolol & derivatives	E	I	ODS	64
Ranitidine & metabolites	H	R	ODS	65
Reserpine	E	F	ODS	66
Spectinomycin	E	S	ODS	67
Sulbenecillin & carbenecillin	M	F	ODS	68
Tamoxifen & metabolites	C	I	ODS	69
Tetracycline & metabolites	M	F	ODS	70
Theophylline & metabolites	M	T	ODS	71
Thioridazine & mesoridazine	E	I	ODS	72
Tryptophan & metabolites	H	V	ODS	73
Vitamins B_1, B_2, B_6	D	F	Phenyl	74

* Key — see Table 7.

‡ Butylsulphonate.

† Key to eluents used — A, water; B, aqueous phosphate buffer; C, 10 mM NaOH in 0.2M Na_2SO_4; D, buffered water–methanol; E, methanol–tetrahydrofuran; F, methanol–water; G, methanol–acetonitrile–buffer; H, methanol–acetic acid–triethanolamine; I, methanol–water–acetic acid; J, acetonitrile–water; K, acetonitrile–acetate buffer; L, water–propan-2-ol; M, methanol–phosphate buffer, N, acetic acid–acetonitrile; P, propan-2-ol/borate buffer; Q, acetonitrile–water–sulphuric acid; R, methanol–water–phosphate buffer; S, acetic acid–sodium sulphate; T, methanol–water–acetate, V, methanol–buffer.

Applications

In an earlier review of the literature pertaining to ion-pair HPLC [21], a listing was presented of all solutes examined using various HPLC systems. Table 8 is intended to be a selective update of that list, and concentrates largely on drug analyses. The significant difference between the present and the earlier list is that current practice recommends almost exclusively the use of a chemically-bonded reversed-phase HPLC system as the basis for development of ion-pair procedures. A further striking feature is the large number of drug ions, of very different physicochemical character, which have been separated by HPLC by using somewhat similar phase systems. This undoubtedly reflects the very flexible nature of the ion-pairing technique.

Concluding Remarks

This contribution has briefly reviewed the uses of ion-pair extraction and high-performance liquid chromatography in pharmaceutical and biomedical analysis. It is hoped that a better appreciation of the principles of the ion-pair effect, and of the rules for its manipulation, will enable the analyst to readily design extraction and HPLC systems for efficient, selective and sensitive analytical procedures. It should be possible to accomplish these objectives without necessarily becoming embroiled in discussions on the putative mechanisms of these processes.

A description of the use of zwitterions, e.g. for the resolution of optical isomers [75], or of hydrophobic metal ions as pairing ions, has not been included in the present work. These techniques and the use of the ion-pair effect in ion-selective electrodes of the liquid membrane type are of some importance in drug analysis, and may be expected to form the basis of subsequent work to be published in the pharmaceutical and biomedical sciences.

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