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RETENTION-OPTIMIZATION STRATEGY FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ION-PAIR SEPARATION OF SAMPLES CONTAINING BASIC COMPOUNDS

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SUMMARY

A simple model is developed to explain sample retention in ion-pair high-performance liquid chromatographic systems when pH is varied. Comparison of the model with literature data shows general agreement. On the basis of this model we propose a retention-optimization scheme for the separation of basic samples via ion-pair chromatography. The data-collection procedure is the same as that described by J. L. Glajch, J. J. Kirkland, K. M. Squire and J. M. Minor [*J. Chromatogr.*, 199 (1980) 57] for four-solvent optimization of reversed-phase separations (mixture-design technique). Application of this approach to several representative samples resulted in rapid optimization of retention and successful separation.

INTRODUCTION

Method development in high-performance liquid chromatography (HPLC) currently emphasizes retention optimization: the control of band positions within the chromatogram so as to maximize sample resolution and/or minimize separation time[1,2]. For the case of non-ionized organic compounds, several groups have shown that variation of mobile phase composition is a powerful and convenient method for optimizing band spacing and resolution[3–9]. This has in turn led to several schemes for systematically varying the kinds and concentrations of various solvents in the mobile phase in order to arrive at a "best possible" separation[6,10–15]. One of these approaches[6,15] has been adapted to commercial hardware so as to allow the automated and unattended development of optimum separation conditions (Sentinel System, DuPont).

Similar procedures have been used for the separation of ionized or ionizable compounds, usually by reversed-phase systems with pH control and/or ion-pairing[16–28]. However these latter separations are less straightforward than those of non-ionized compounds by reversed-phase systems, particularly for samples that contain basic compounds. Thus, basic compounds often exhibit tailing and greater column-

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to-column variations in retention. The separations themselves are more complex because of the large number of variables that significantly affect band spacing: the kinds and concentrations of organic solvents, ion-pairing reagents, basic anti-tailing compounds, buffers and salts in the mobile phase, pH of the mobile phase and separation temperature. Finally, changes in retention as a result of some change in separation conditions are less predictable than for the reversed-phase separation of non-ionic compounds, and this often necessitates a trial-and-error search for the right solvent strength.

Another problem in optimizing ion-pair separations is their theoretical complexity. A number of mathematical treatments of retention *vs.* separation conditions have been offered[3,29–33], but these are generally rather complicated and do not treat the separate effects of all the major variables. Here we will present a simplified but comprehensive model that allows ready visualization of the essential features of these separations. The model in turn suggests a simple approach to retention optimization in ion-pair systems, one that seems to solve typical separation problems in a relatively short time. This approach is also open-ended in its ability to handle problems of greater difficulty by invoking additional optimizable variables as needed. Several applications of this approach will be illustrated as carried out on the same Sentinel System as that used for retention optimization of non-ionic samples.

THEORY

On the basis of both theory and experience there is general agreement that pH variation and the use of ion-pairing typically lead to major changes in band spacing and resolution. However, we have noted a number of other separation variables that also affect the separation of basic compounds, and previous workers[16–28] have at one time or another made use of all of these variables. The following theoretical treatment suggests a simple approach to the optimization of retention for basic compounds.

Retention in ion-pair systems

We begin with the model of Knox and Hartwick[31], which has been adequately verified for ion-pair systems, and expand it to include reversed-phase retention of non-ionized compounds, as well as pH effects. The relevant equilibria are: (1) sorption of ion-pair reagent P^-M^+ onto the surface S of the column packing (eqn. 1); (2) ionization of the solute X (eqn. 2); (3) solute ion exchange (eqn. 3); (4) reversed-phase sorption of solute (eqn. 4). These processes are described by the following expressions:

$$P^- + M^+ + S \rightleftharpoons PMS \qquad K_{ps} = \theta_p/[P^-]\,[M^+]\,\theta_s \tag{1}$$

$$XH^+ \rightleftharpoons X + H^+ \qquad pK_a = [X]\,[H^+]/[XH^+] \tag{2}$$

$$XH^+ + PMS \rightleftharpoons M^+ + PXHS \qquad K_{ie} = [M^+]\,[PXHS]/[XH^+]\,\theta_p \tag{3}$$

$$X + S \rightleftharpoons XS \qquad K_{xs} = [XS]/[X]\,\theta_s \tag{4}$$

Here θ_p refers to the fraction of the column-packing surface covered by adsorped

pairing ion P^-M^+, and $\theta_s = (1 - \theta_p)$ is the fraction of uncovered surface. The fraction of solute molecules in the non-ionized (θ_x) and ionized (θ_{x+}) forms can be derived:

$$\theta_x = [X]/([X] + [XH^+]) = 1/(1 + [H^+]/K_a) \tag{5}$$

and

$$\theta_{x+} = 1 - \theta_x = 1/(1 + K_a/[H^+]) \tag{6}$$

The capacity factor k' is the sum of reversed-phase and ion-pair retention processes, or

$$k' = \psi([PXHS] + [XS])/([X] + [X^+]) \tag{7}$$

where ψ is the phase ratio (V_s/V_m), and V_s and V_m refer to the volumes of stationary and mobile phases within the column. Combination of eqns. 1–7 then yields

$$k' = \psi \left\{ \left(\frac{K_{ie}\, \theta_{x+}\, \theta_p}{[M^+]} \right) + (K_{xs}\, \theta_x\, \theta_s) \right\} \tag{8}$$

(i) (ii)

Term i of eqn. 8 represents the ion-pair retention of XH^+, while term ii accounts for the reversed-phase retention of non-ionized X. The fractional coverage of the stationary phase surface by ion-pair reagent (θ_p) can be obtained from eqn. 1:

$$\theta_p = K_{ps}\,[P^-]\,[M^+]/1 + k_{ps}\,[P^-]\,[M^+] \tag{9}$$

This is, we assume a Langmuir isotherm for uptake of ion-pair reagent by the stationary phase.

For systems with significant concentrations of buffer and/or neutral salts, in addition to pairing ion, the term $[M^+]$ can be taken to mean the *effective* concentration of all cations in the system. The significance of eqn. 8 is illustrated in Fig. 1 for a model ion-pair system, where k' is plotted *vs.* pH. Conditions (see Fig. 1) have been chosen to yield $\theta_p = \theta_s = 0.5$. At pH values below pK_a, $\theta_{x+} > \theta_x$, ion pairing is the dominant process (term i of eqn. 8). Likewise, at higher pH values, reversed-phase retention is more important. Because k' decreases at higher pH in this example, ion-pair retention can be concluded to be stronger than reversed-phase retention, *i.e.*, $K_{ie} > K_{xs}$. The general shape of Fig. 1 (k' *vs.* pH) will be retained as the concentration of ion-pair reagent is varied, or for compounds having different reversed-phase (K_{xs}) or ion-pairing (K_{ie}) tendencies. However, the limiting k' values at high and at low pH will shift accordingly.

Selectivity effects embodied in eqn. 8. Consider next the consequences of eqns. 8, 9 with regard to the dependence of band spacing on experimental conditions and solute structure. In a following section we will consider the effects of other variables (not included in eqn. 8) on bandspacing. The pertinent solute characteristics are pK_a

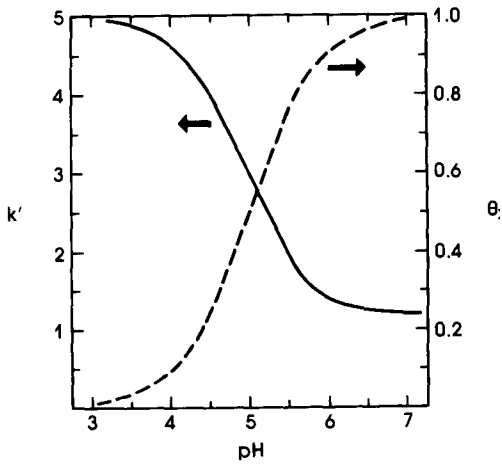

Fig. 1. Variation of retention in ion-pair chromatography with pH. Calculation from eqn. 8, with conditions as follows: $K_{ie} = 5$, $K_{xs} = 1$, $K_{ps} = 250$, $pK_a = 5$, buffer concentration $[M^+]$ equal 200 m*M* and ion-pairing reagent concentration $[P^-] = 20$ m*M*. (——) k' Values; (---) fraction of solute X in non-ionized form (θ_x).

value and the net charge on the solute molecule when fully ionized. Relevant experimental conditions include: concentration of the ion-pair reagent, its binding affinity (K_{ps}), pH, and the kinds and concentrations of various salt and buffer species (represented by $[M^+]$).

Eqns. 1–8 can be expanded to include the case of multiply-charged solutes. For example, a solute capable of existing as either Y, YH^+ of YH_2^{2+} leads to the following relationships (*cf.* eqns. 3, 8):

$$YH_2^{2+} + 2PMS \rightleftharpoons 2M^+ + YH_2P_2S_2$$

$$K_{ie2} = [M^+]^2\,[YH_2P_2S_2]/[XH_2^{2+}]\,[PMS]^2 \tag{10}$$

and

$$k' = \psi\{(K_{ie2}\,\theta_{y^{2+}}\,\theta_p^2/[M^+]^2 + K_{ie}\,\theta_{y^+}\theta_p)/[M^+] + K_{ys}\theta_y\theta_s\} \tag{11}$$

Here, $\theta_{y^{2+}}$, θ_{y^+} and θ_y refer to the fractions of Y in the 2+, 1+ and uncharged forms, respectively, in the mobile phase.

Consider first the effect of the ion-pair reagent on the relative separation (α) of two solutes, X and Y. The kind of ion-pair reagent chosen (*e.g.*, pentanesulfonate, heptanesulfonate, etc.) determines the value of K_{ps}, and the effect of the pairing ion can be further adjusted by varying its concentration $[P^-]$. However, eqns. 8 and 11 show that k' depends only on the surface coverage of the stationary phase by pairing ion (θ_p), which is determined (eqn. 9) by the product $K_{ps}[P^-][M^+]$. Thus, a higher concentration of a less strongly sorbed pairing ion (*e.g.*, pentanesulfonate) should be equivalent to a lower concentration of a more strongly retained pairing ion (*e.g.*, heptanesulfonate). Knox and Hartwick[31] have shown this to be the case for retention

of solutes of varying charge (−1, 0 and +1), using C_8, C_{10} and C_{12} sulfates as pairing ions. *Thus in principle, a single pairing ion can provide all the selectivity available from a range of different pairing ions, providing that its concentration can be varied to change θ_p over a wide range.* This means that a single ion-pair reagent can be selected for retention-optimization in ion-pair chromatography, the choice of pairing-ion depending on practical considerations (ease of removing the pairing ion from the column between different optimization experiments, solubility, availability, etc.).

An increase in $[P^-]$ leads to an increase in θ_p and predictable changes in solute band-spacing. According to eqn. 11, solute retention should increase with θ_p^2 for double-charged solutes, with θ_p for singly-charged solutes, and with $(1 - \theta_p)$ for uncharged solutes*. Qualitatively, this has been observed[22] for the separation of phenols, phenol monosulfonates and phenol disulfonates by ion-pairing with tetraalkyl ammonium ions. As either K_{ps} or $[P^-]$ increases, the monosulfonates are more retained than phenols, and disulfonates are more retained than monosulfonates.

For the case of compounds X and Y having different pK_a values, eqn. 6 indicates that their θ_{x+} and θ_{y+} values will also differ. This then leads to differences in relative retention as θ_p is varied (by changing $[P^-]$). Thus, if $(pK_a)_x > (pK_a)_y$ (X is more basic), $\theta_{x+} > \theta_{y+}$. Therefore, as θ_p is increased via increase in $[P^-]$, this will result in a greater increase in k' for solute X than for solute Y (eqn. 8). That is, a change in band spacing will result. As in the case of reversed-phase retention of acids or bases without ion-pairing[3], the maximum change in band spacing will occur at a pH intermediate between $(pK_a)_x$ and $(pK_a)_y$. This variation of band spacing with change in θ_p for solutes of varying pK_a has been demonstrated in the separation of pafenolol and five related compounds with dimethylcyclohexylsulfonate (DMCS) as pairing ion[21]. These six compounds can be grouped into neutral, weakly basic (–CH(OH)–CH_2–N–), and basic (–CH_2–CH_2–N–) classes; *i.e.*, with pK_a values increasing in this order. As DMCS concentration is increased, the retention of the basic compounds increases markedly, the retention of the weakly basic compounds increases slowly, and the retention of the neutral solutes decreases moderately, in agreement with eqn. 8. The degree of change is also predicted to vary with pH, but this was not studied by Jannson and Johansson[21].

Consider next the effect of buffer concentration on band spacing. The general effect can be seen more easily in eqn. 8, which shows that retention decreases with an increase in $[M^+]$. However, this effect is more important for higher concentrations of buffer and ion-pair reagent (see eqn. 9); *i.e.*, when θ_p is fairly large. At low values of θ_p, a change in buffer concentration will have less effect on either retention or band spacing. From eqn. 11 it can be seen that the effect of an increase in buffer concentration $[M^+]$ will be larger for solutes of higher charge. Thus, when θ_p is significant (*e.g.*, > 0.2), an increase in buffer concentration will cause a larger decrease in retention for XH_2^{2+} *vs.* XH^+, or for XH^+ *vs.* X. These changes in band spacing are similar to those achievable via change in $[P^-]$ and θ_p. *Therefore changes in buffer concentration duplicate the result of change in pairing-ion concentration, and add little to the control of retention in ion-pair chromatography systems.* (See also the examples and discussions in refs. 3, 19, 23–25 and 34.)

* With suitable change in notation, eqns. 1–11 apply to negatively charged solutes (X^-, X^{2-}) and positive pairing ions (P^+) as well.

Other selectivity effects. The above discussion confirms the importance of pH and ion-pair reagent concentration as variables of primary importance in controlling band spacing. Experience also indicates that the same variables can be quite useful in optimizing band shape (reducing band tailing, increasing band sharpness), and for these reasons we have selected pH and pairing-ion concentration as initial variables in our retention-optimization scheme. However, if further optimization of band-spacing is required after variation of pH and $[P^-]$, other variables can be examined.

Solvent selectivity. Variation of the organic solvent used to control solvent strength (methanol, acetonitrile, tetrahydrofuran) for reversed-phase separations of non-ionic species can have a major effect on band-spacing[4–8]. Similar effects can be anticipated in the separation of ionizable solutes, and limited studies confirm this[24,27,35,40]. In this study we explored the possibility of changing the organic solvent in order to provide further changes in band-spacing, in addition to those provided by pH and $[P^-]$ variation.

When changing the organic solvent or its concentration Φ in ion-pair chromatography, it should be noted that θ_p decreases with increasing solvent strength[36]. That is, less of the ion-pair reagent is adsorbed, and the ion-pairing effect (term i of eqn. 8) is thereby reduced. We recommend that solvent strength be held constant during attempts at changing band-spacing, which makes this effect of less practical importance.

Temperature effects. A change in temperature for reversed-phase systems and non-ionized solutes generally has little effect on band-spacing[37–39]. The reason is that a single retention process (hydrophobic binding of solute to the stationary phase) is usually involved, and in this case enthalpies of retention are often correlated with entropies of retention. The result is that more strongly retained solutes have larger retention enthalpies and larger changes in retention with temperature, but compounds that are adjacent within the chromatogram (similar retention) then exhibit similar retention variations with change in temperature, and no change in band spacing.

As indicated in eqns. 1–4, several different processes are normally involved in ion-pair chromatography: sorption of pairing ion, acid–base equilibria of the solutes, solute ion exchange, and hydrophobic (reversed-phase) binding of solute to the stationary phase. Each of these steps can have differing enthalpies of reaction, so that the final equation for retention (eqn. 8) is a complex function of several enthalpy terms. In this situation it is unlikely that the overall temperature coefficient of retention will be closely correlated with absolute solute retention, except in the case of solutes of very similar structure. As a result, it is observed[24,27,35] that a change in temperature often results in changes in band-spacing for ion-pair systems. Such effects are predicted to be largest when values of θ_p and θ_x are equal to about 0.5, but less important for $\theta_p \approx 0$ or 1. Limited data appear to show minimal band-spacing changes under the latter conditions[28].

Second-order effects. There are a number of documented phenomena not encompassed in the model of eqn. 8. While we believe these are of little general importance as far as band spacing is concerned, they can have an appreciable effect on solute retention in certain systems. Eqn. 8 is therefore at best a semi-quantitative relationship, better suited for picturing the important retention processes than for fitting actual experimental data. One such effect is an apparent increase in solvent

strength with increase in $[P^-]$[32]. A similar increase in solvent strength by increasing the concentration Φ of organic solvent usually has only a minor effect on band-spacing[37]. The present model ignores changes in activity coefficients for various electrolytes, as ionic strength and other conditions are varied. These effects have been discussed in detail by Karger *et al.*[3], but their practical significance is still uncertain.

Certain other processes are also known to contribute to retention in many ion-pair systems: (a) ion exchange with accessible silanols in the bonded-silica packing[41]; these effects are minimal at higher ionic strengths and in the presence of ion-pair reagent; (b) silanophilic retention of bases on accessible silanols[39,42]; this is much reduced in the presence of added amines, as in the present study; (c) hydrophobic binding of charged species XH^+ to the uncovered stationary phase surface (with volume θ_s); this should be minor in ion-pairing systems, particularly where $\theta_x \neq 0$; (d) sorption of solute X onto the surface covered by adsorbed pairing-ion P^-; this is suggested by the ability of conventional ion exchangers to bind non-ionic species, such as sugars. We can accomodate this latter effect into eqn. 8

$$k' = \psi \frac{K_{ie}\,\theta_{x^+}\,\theta_p}{[M^+]} + K_{xs}\,\theta_x\,\theta_s + K'_{xs}\,\theta_x\,\theta_p \qquad (12)$$

Here K'_{xs} represents the equilibrium constant (as for eqn. 4) for sorption of X onto the surface covered by P^-. Generally, we should find that $K'_{xs} \ll K_{xs}$, so that the last term of eqn. 12 is relatively unimportant.

EXPERIMENTAL

A Sentinel System (DuPont, Wilmington, DE, U.S.A.), consisting of a four-solvent pump and controller, video display module, heated column compartment, spectrophotometric detector, auto sampler and data system, was equipped with a Zorbax® C_8 column. Organic solvents and buffer salts were from Fisher Scientific (Pittsburgh, PA, U.S.A.) and J. T. Baker (Phillipsburg, NJ, U.S.A.). Ion-pair reagents were purchased from Eastman-Kodak (Rochester, NY, U.S.A.). Purified water was obtained from a laboratory water-purification system (Milli-Q from Millipore, Bedford, MA, U.S.A.). Unless stated otherwise, all separations were carried out at a temperature of 50°C and a mobile phase flow-rate of 3 ml/min.

The approach used here was as described previously[15] for four-solvent optimization in reversed-phase separations. Solvent strength was adjusted manually to maintain constant running time (constant k' value for last peak) as discussed in the following section. Column lifetime was initially observed to be impractically short, presumably because of the use of high pH values (7.7) and higher ionic strength for some mobile phases. Other experimenters[43] have also noted that the combination of low pH ($\leqslant 2$) with methanol–water mobile phases appears to shorten the life of reversed-phase columns. We then added a 25-cm length of column, packed with silica between the pump and sample valve. Subsequent operation with this "silica-saturated" system resulted in column life-times that were comparable to those in other HPLC systems.

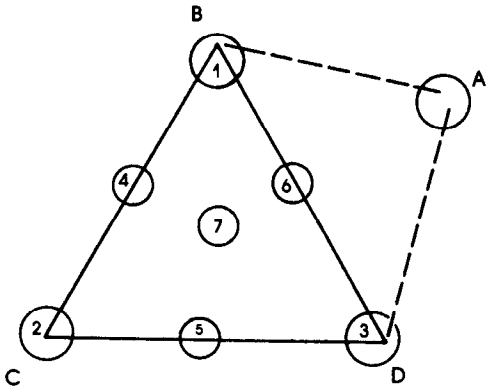

Fig. 2. The basis of four-solvent, seven-mobile-phase optimization as used in the present study. See Table I for compositions of solvents A–D.

RESULTS AND DISCUSSION

The method-development or retention-optimization approach used by us is illustrated in Fig. 2, which can be compared with the similar approach for four-solvent reversed-phase optimization in refs. 6 and 15. Seven mobile phases are selected to map the effect of pH and of ion-pair reagent concentration on the retention of each sample component. The three selectivity solvents from the corners of the triangle B–D (or Nos. 1–3) are described in detail in Table I. Methanol (the fourth solvent, A) is added to adjust the solvent strength so as to give a constant k' value for the last band in the chromatogram. Solvents 1 and 2, 2 and 3, and 3 and 1 are blended in a ratio of 1:1 by volume to give mobile phases 4, 5, and 6 respectively. Mobile phase No. 7 is a 1:1:1 blend of solvents 1–3. The optimization procedure is begun with a linear gradient running from 100% solvent 1 to 100% methanol (3 ml/min, 20 min). From the retention time of the last sample band, an estimate is made of the vol. percentage methanol required in solvent 1 in order to achieve a reasonable k' value for the last band in an isocratic chromatogram (*e.g.*, $k' = 8$; *cf.* discussion of p. 693 of ref. 1 and ref. 44). Mobile phase of this composition is then blended and used in an isocratic separation of the sample. If adjustment of solvent strength is required to achieve the predetermined k' value for the last band, this is done by trial and error (p. 53 of ref. 1). Use of the relationship

$$\log k' = \log k_0 - S\,\Phi \tag{13}$$

TABLE I

COMPOSITIONS OF STANDARD FOUR MOBILE PHASES DESCRIBED IN FIG. 2

Mobile phase	*Composition*
A	Methanol
B	100 m*M* Citric acid, 20 m*M* triethylamine, pH 2.5
C	100 m*M* Citric acid, 20 m*M* triethylamine, pH adjusted to 7.5 with sodium hydroxide
D	200 m*M* Hexanesulfonic acid, 20 m*M* triethylamine, pH adjusted to 5.4 with citric acid

is useful in this estimation process; Φ is the volume fraction of methanol in the mobile phase, S is a constant for a given solute (usually $3 \leqslant S \leqslant 6$) and k_0 is the value of k' for water as mobile phase. This process is repeated for solvent 2, starting with the final value of Φ for the run with solvent 1, then extended to solvent 3. Once the solvent strengths (value of Φ) for solvents 1–3 have been adjusted for constant k' of the last band, solvents 1–3 are blended as above to obtain solvents 4–7. The sample is then chromatographed with these seven solvents, as are the individual compounds in the sample. The results are processed by the Sentinel System to provide an overlapping-resolution map[6]. Alternatively, gradient elution with solvents 2 and 3 as starting solvent could also be used to estimate the best value of Φ in the corresponding isocratic elution.

Mobile phase compositions

The choice of conditions in Table I deserves comment. Citrate was chosen as buffer for its ability to provide a roughly linear variation of pH for blends of solvents 1 and 2. For 0–95% (v/v) solvent 1 in mixture with solvent 2, the deviation of pH from a linear change with volume fraction of solvent 1 was less than 0.1 pH unit. It has also been noted[23] that citrate gives a better peak shape than with phosphate buffers in reversed-phase separations with pH and ionic strength variation. The choice of ion-pair reagent represents a compromise between two factors: (1) longer-chain alkyl sulfonates give a larger ion-pair effect with lower concentrations of ion-pair reagent[22]; (2) longer-chain sulfonates are much more difficult to wash from the column[31]. On the basis of these considerations we selected hexane sulfonate as reagent. In optimization studies such as these it is particularly important that a column can be rapidly equilibrated with a new mobile phase, following use of the preceding mobile phase. The choice of reagent concentration in solvent 3 was a compromise between solubility considerations (in mobile phases of different methanol content) and maximum ion-pairing effect. Fig. 3 shows a plot of retention (proportional to ion-pairing effect, see eqn. 8) *vs.* the concentration of hexanesulfonate. While a maximum in k' is achieved at 300 m*M* reagent, we chose 200 m*M* for solvent 3 to avoid solubility problems.

The mobile phases of Table I are seen to contain 20 m*M* of triethylamine (TEA) in order to suppress band-tailing[21]. The concentration of the TEA should be much less than that of pairing-ion in solvent 3, but should be large enough to suppress unwanted silanol interactions. The 20 m*M* concentration selected was based on experiments such as those shown in Fig. 4. We also wondered whether the reduction in band asymmetry from added TEA did not simply reflect the lower k' values obtained upon addition of TEA. Parallel experiments with fixed TEA concentration but varying methanol content showed that this was not the case.

The concentration of citrate selected (100 m*M*) was based on experiments of pH linearity, as discussed above (*i.e.*, adequate buffering capacity). Thus blends of solvents 2 and 3 gave linear plots of pH *vs.* volume fraction of solvent 2, from 0–90% solvent 2 (± 0.05 units). We were also concerned about lower concentrations of the buffer causing band-tailing as in ref. 31.

Methanol was chosen as organic solvent, primarily because solubility problems with the various buffers and reagents were less than with other solvents. When detection in the low UV region (< 220 nm) is desired, phosphate buffer can be used in place of citrate. However, we have not explored this possibility.

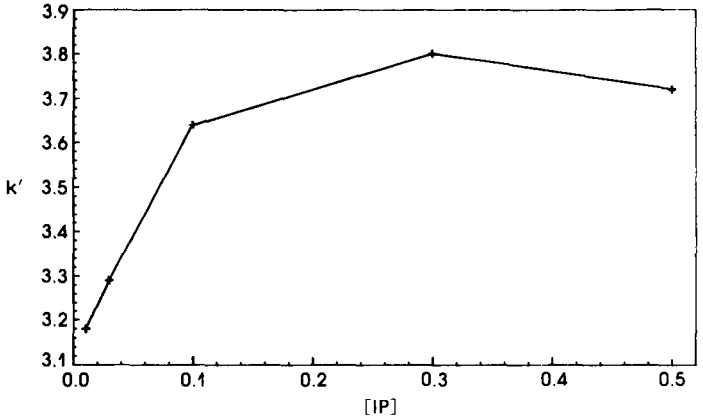

Fig. 3. Retention (k') of 2,4-dimethylanaline solute *vs.* Concentration of hexanesulfate ([IP]). Sodium acetate concentration = 0.4 m*M*; pH = 3.6; 45% methanol; Zorbax C_8 column.

Applications of the present procedure

Additives in cough syrup. The compounds phenylephrine, glyceryl guiacolate, pseudoephedrine, sodium benzoate and methyl paraben are common constituents of various cold remedies. Therefore these five compounds were selected as candidates for the present scheme. Note that, in addition to the basic compounds, phenylephrine and pseudoephedrine, the mixture contains an acid (benzoate) and two neutral species. The resulting separations with solvents 1–7 are shown in Fig. 5). These results were used to generate an overlapping-resolution map (ORM), shown in Fig. 6a, and the optimum mobile phase indicated by that map was used for the optimized separation of Fig. 6b. While the resolution of Fig. 6b is quite acceptable, note that benzoate (peak 4) is eluted near t_0. Generally, this is undesirable for quantitation, because interference by peaks eluted near t_0 are likely. The problem here is that mixtures of acids and bases are difficult to separate with ion-pair chromatography, because either the acidic or basic compounds will tend to be eluted early (see discussion of ref. 31). However, the present format (Figs. 5 and 6a) often offers an

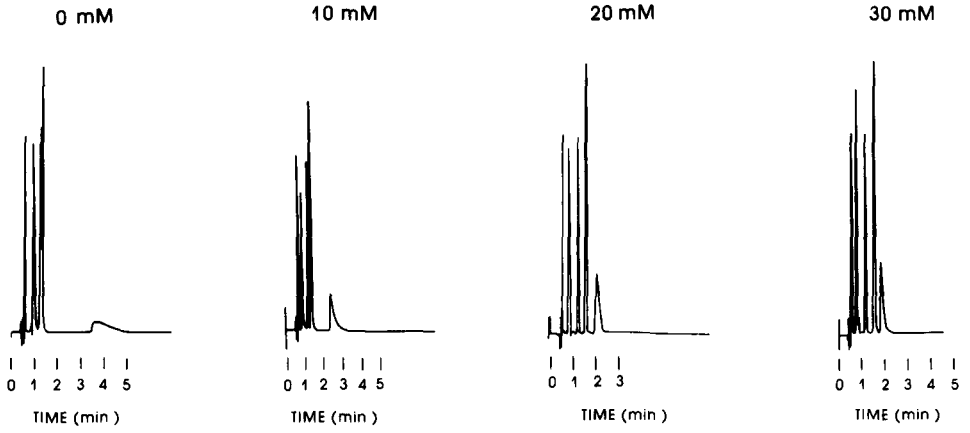

Fig. 4. Band shape of 2,4-dimethylamine solute *vs.* concentration of triethylamine added to mobile phase.

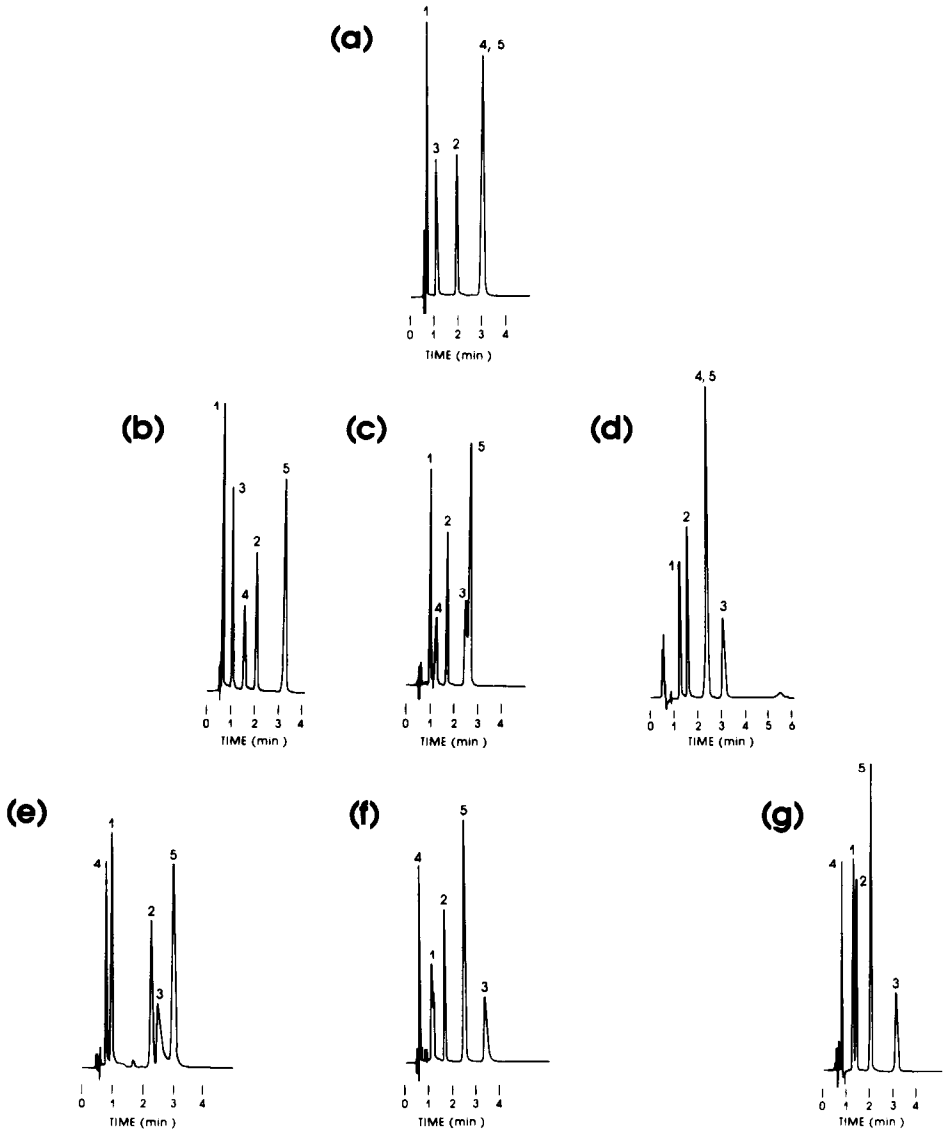

Fig. 5. Optimization of retention for five compounds used in cough remedies: 1 = phenylephrine; 2 = glycerol guaicolate; 3 = pseudoephedrine; 4 = sodium benzoate; 5 = methylparaben. Conditions: Zorbax C_8 column (15 × 0.46 cm I.D.), flow-rate 3 ml/min, temperature 50°C. Mobile phase compositions (a–g in Fig. 5 correspond to solvents 1, 4, 7, 6, 2, 5 and 3 of Fig. 2, respectively): (a) 30% A, 70% B (see Table I); (b) 28.6% A, 35% B, 36.4% C; (c) 30.4% A, 23.3% B, 24.2% C, 22.1% D; (d) 31.8% A, 35% B, 33.2% D; (e) 27.3% A, 72.7% C; (f) 30.4% A, 36.4% C, 33.2% D; (g) 33.7% A, 66.3% D.

acceptable compromise for problems such as this. That is, other compositions (lighter regions of Fig. 6a) indicate better resolution, even if less than the optimum of Fig. 6b. We can consider these other compositions while looking for conditions where no band is eluted at t_0. One such combination is suggested by Fig. 5c (solvent 7), where addition of a small amount of solvent 4 should simultaneously yield acceptable reso-

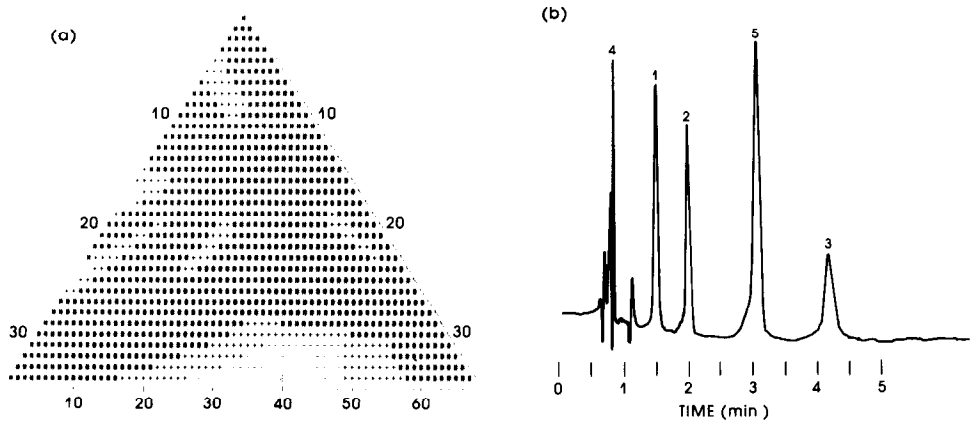

Fig. 6. Separation of cough-remedy compounds. (a) Overlapping-resolution map (ORM) for chromatograms in Fig. 5a; (b) optimum separation predicted by ORM; conditions as in Fig. 5, except for mobile phase: 31.1% A, 29.7% C, 39.2% D.

lution and no bands at t_0. This prediction is confirmed in Fig. 7. Likewise, if the bands in Fig. 6b exhibited tailing, other compositions with improved band shape could have been tried.

Water-soluble vitamins. A mixture of vitamin C, pyridoxine, niacinamide, thiamin, and riboflavin was used as a sample for this optimization series. Figs. 8a–c show the results for solvents 1–3, Fig. 8d shows the ORM obtained with the standard seven mobile phases, and Fig. 8e shows the optimum separation, which in this case requires a blend of all four solvents. As in the previous example, one compound (vitamin C) is eluted near t_0. However, in this case this was true for all seven mobile phases.

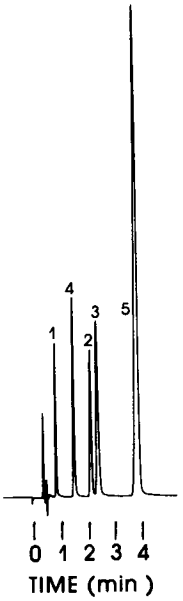

Fig. 7. Separation of cough-remedy compounds with sup-optimum resolution but no compounds eluted at t_0. Conditions: same as in Fig. 5, except for mobile phase: 30.4% A, 25.2% B, 27.8% C, 16.6% D.

Thus, no isocratic mobile phase composition that simultaneously provides adequate resolution and significant retention of all five sample compounds could be found.

Tricyclic antidepressants. Six compounds from this group were selected for the present optimization procedure: amitryptiline, nortryptiline, imipramine, desipramine, doxepin and protryptiline. Figs. 9a–c show the separation of this mixture in solvents 1–3, Fig. 9d shows the ORM for all seven solvents, and Fig. 9e shows the resulting optimum separation. Although this final separation is acceptable, this sample was chosen to explore the possibility of further optimizing the separation by changing the organic solvent. The solvent methanol of Table I was replaced by acetonitrile and then by tetrahydrofuran (THF) to generate fourteen additional mobile phases and two ORMs with resulting optimized separations. The ORMs are shown in Figs. 10a (acetonitrile) and 10c (THF), while the optimized separations are shown in Figs. 10b (acetonitrile) and 10d (THF). Neither separation is as good as that with methanol in Fig. 9e, and the THF system shows marked tailing. It would be wrong, however, to conclude that acetonitrile and THF are not useful solvents in this optimization approach. Significant differences in band-spacing were produced by the use of these solvents *vs.* methanol, as summarized in Table II. Other samples, showing poorer separation when optimized with methanol as organic solvent, may well show improved separation with acetonitrile or THF.

The previous example raises the question of how method development or retention optimization should proceed if unsatisfactory results are obtained by the present procedure (with methanol as organic solvent). It is not possible to answer this question on the basis of present experience, but we favor the following strategy:

(1) If large changes in k' occur with change in pH (solvents 1 *vs.* 3) and the optimum pH from the ORM is $(pH)_{opt}$, then repeat the entire process, but replace solvents 1 and 2 with equivalent buffers having pH values equal to $[(pH)_{opt} - 1]$ and $[(pH)_{opt} + 1]$, respectively. Some researchers[25] have argued that pH optimization requires small changes (± 0.2 units) in pH for the various mobile phases used in the study.

(2) If large changes in k' with change in pH are not found, try changing the organic solvent as in the studies of Figs. 9, 10.

(3) If adequate separation is not found at this point, examine the results from the preceding step to see if some mixture of the three organic solvents might provide improved separation.

(4) If further variation in band-spacing is needed, repeat the seven-mobile phase optimization at a different temperature (*e.g.*, 30 *vs.* 50°C) or with a different column (*e.g.*, cyano-silica or phenyl-silica; *cf.* ref. 44). Normally, it is advantageous to operate at a temperature of 50–60°C (*cf.* discussion of ref. 37), but marked changes in band-spacing with small change in temperature are possible.

Mixture of aromatic amines. As a final example for a mixture of model aromatic amines, a six-compound mixture was selected: *p*-chloroaniline, 2,4-dinitroaniline, 3,4-dichloraniline, 4-chloro-2-nitroaniline, 2,5-dichloroaniline and 2,6-dichloro-4-nitroaniline. The results are summarized in Fig. 11: separation with solvents 1–3 in Figs. 11a–c, the seven-mobile phase ORM in Fig. 11d and the optimum separation (same as Fig. 11a). In this case, the amines are only weakly basic, and also generally similar in chemical structure. Consequently no inversions of band position occur, but nevertheless resolution varies significantly as a function of mobile phase composition.

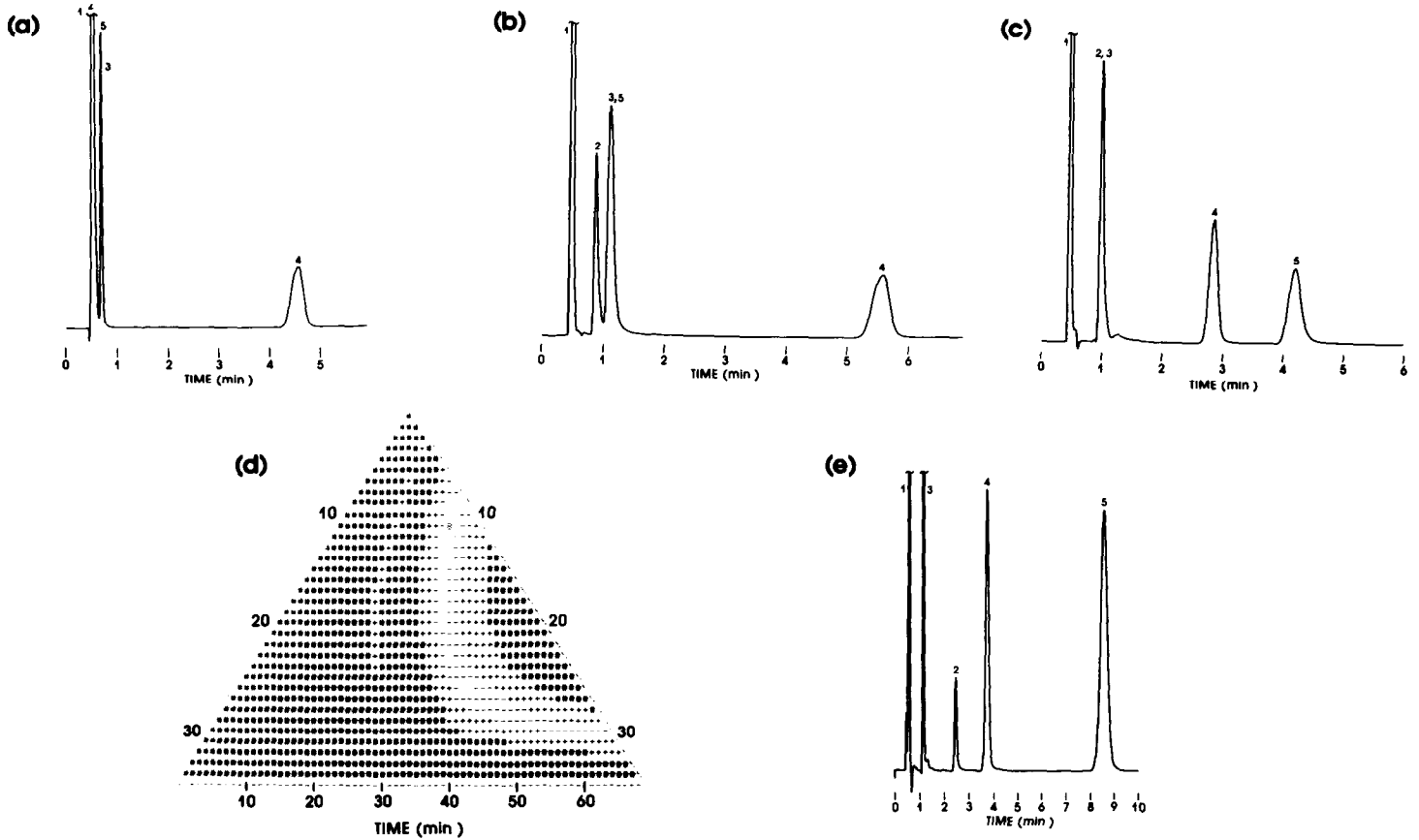

Fig. 8. Optimization of retention for five water-soluble vitamins. 1 = Vitamin C; 2 = pyridoxine; 3 = niacinamide; 4 = riboflavin; 5 = thiamine. (a) Separation with mobile phase 1 (14.6% A, 85.4% B); (b) separation with mobile phase 2 (14% A, 86% C); (c) separation with mobile phase 3 (8.5% A, 91.5% D); (d) ORM for seven-mobile phases; (e) optimum separation predicted by ORM (13.1% A, 59.5% B, 5.2% C, 22.2% D). Other conditions as in Fig. 5.

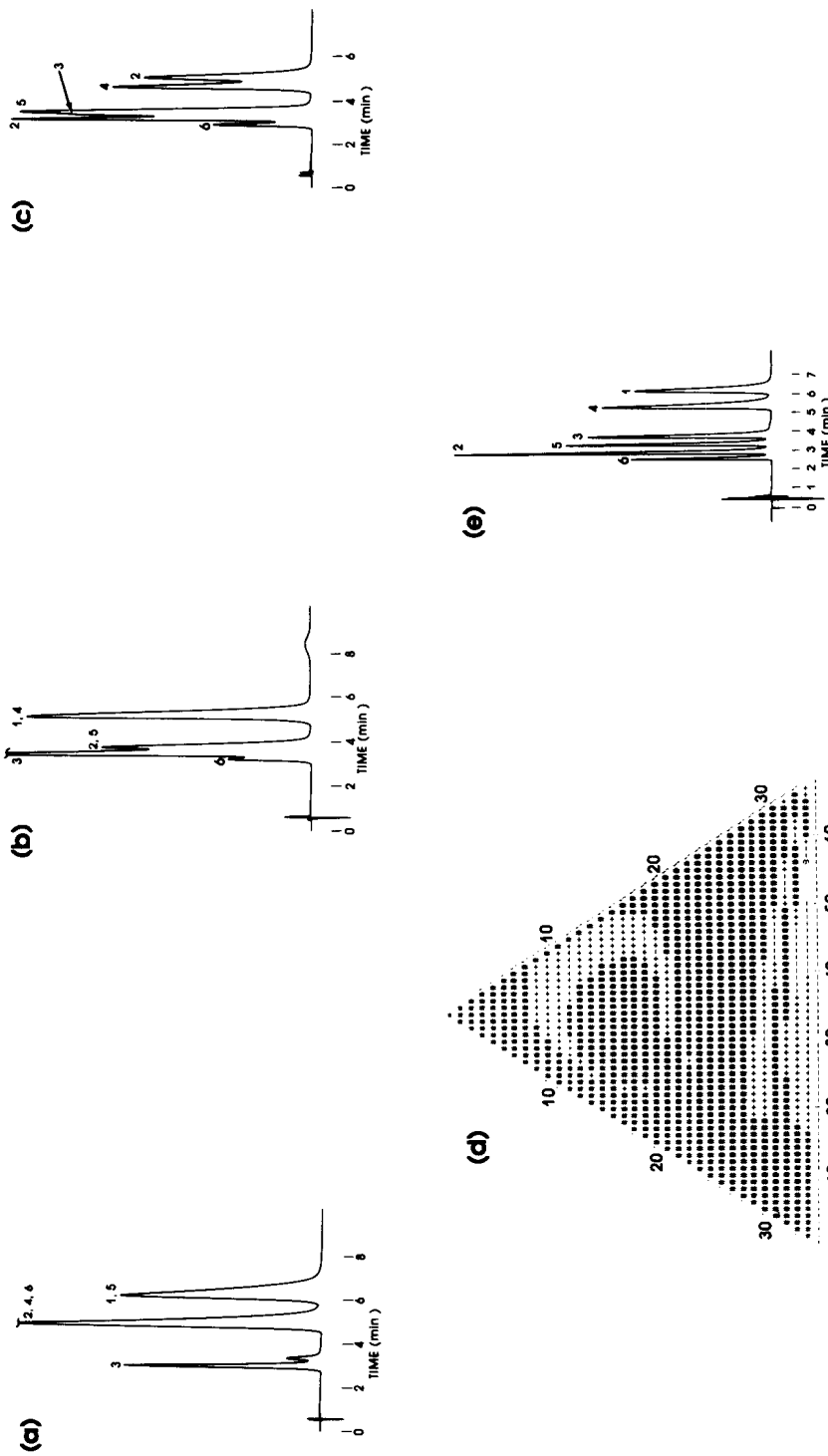

Fig. 9. Optimization of retention for six tricyclic antidepressant drugs. 1 = Amitryptiline; 2 = desipramine; 3 = doxepin; 4 = imipramine; 5 = nortryptiline; 6 = protryptiline. (a) Separation with mobile phase 1 (43% A, 57% B); (b) separation with mobile phase 2 (75% A, 25% C); (c) separation with mobile phase 3 (65% A, 35% D); (d) ORM for seven mobile phases; (e) optimum separation predicted by ORM (66.7% A, 4.1% C, 29.2 D). Other conditions as in Fig. 5.

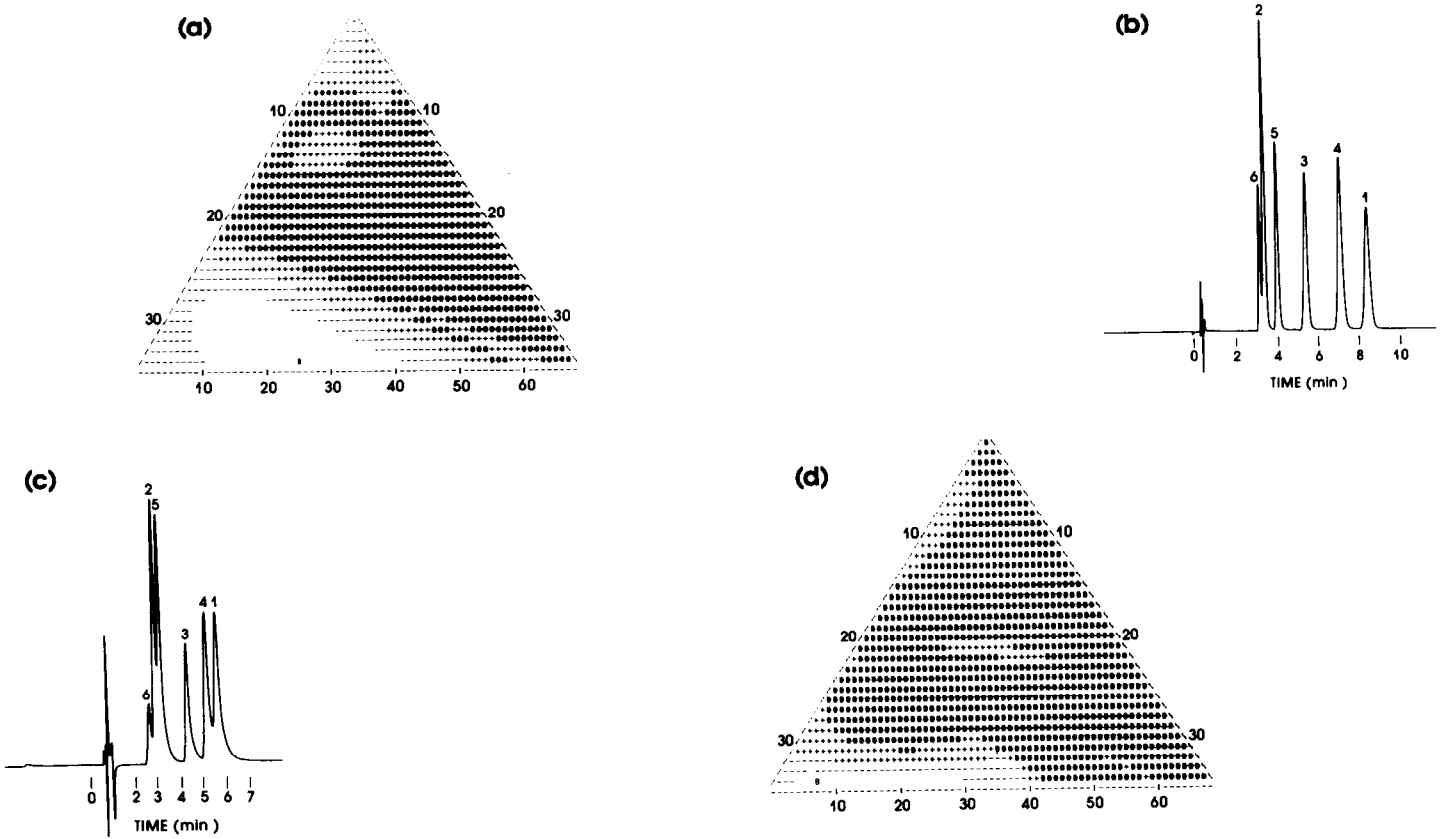

Fig. 10. Combination of pH/ion-pairing optimization with organic-solvent optimization for tricyclic antidepressant drugs of Fig. 9. (a) ORM for acetonitrile as organic solvent; (b) optimum separation predicted by ORM of (a) (52% acetonitrile, 25.5% C, 22.5% D); (c) ORM for THF as organic solvent; (d) optimum separation predicted by ORM of (c) (34.3% THF, 58.6% C, 7.1% D). Other conditions as in Fig. 9.

TABLE II

VARIATION OF SEPARATION FACTORS α WITH pH, ION-PAIR REAGENT CONCENTRATION AND ORGANIC SOLVENT FOR TRICYCLIC ANTIDEPRESSANT DRUGS

Band pair	*Values of* α*								
	Methanol			*Acetonitrile*			*Tetrahydrofuran*		
	1	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*
Amitryptiline/nortryptiline	1.04	1.38	1.52	1.16	1.74	1.34	0.82	1.87	0.91
Nortryptiline/imipramine	1.26	0.74	0.74	1.07	0.66	0.88	1.48	0.58	1.23
Imipramine/desipramine	1.02	1.47	1.55	1.20	1.84	1.36	0.84	1.92	0.90
Desipramine/protryptiline	1.00	1.10	1.11	0.95	1.04	1.02	0.95	0.93	1.07
Protryptiline/doxepin	1.84	0.96	0.85	1.50	0.75	1.10	1.84	0.69	1.39

* Numbers for each solvent refer to solvents 1–3 of Fig. 2.

The marginal (but optimum) separation of Fig. 11a with this 6-μm column could be significantly improved with no increase in separation time by exchanging the latter column for an equivalent 3-μm column, as shown in Fig. 11e (Golden Series® C_8; DuPont; see Discussion of ref. 45).

Other considerations

It is essential in method development procedures such as the present one to equilibrate the column thoroughly with each new mobile phase before retention data are collected. We observed that column equilibration generally required passage of 12–25 column volumes of new mobile phase before k' values became reproducible. We therefore routinely flushed the system with 25 volumes of new mobile phase when changing from one mobile phase composition to the next. We also observed that the usual 2-μm filters ("sinkers") used to filter solvents entering the pump tend to accumulate residues that contribute to retention irreproducibility during method development as described here. We therefore omitted these filters from the system and filtered all solvents through 0.5-μm filters prior to use.

We have commented on the problem of predicting the correct solvent strength for mobile phases 2–7 during retention optimization. The trial-and-error approach (manual mode) described here can easily be adapted to the Sentinel System for fully automated and unattended method development. However, this commonly results in a considerably larger number of experiments (more than seven). An alternative approach is to use gradient elution during method development; *i.e.*, to carry out all seven experiments in a gradient mode.

CONCLUSIONS

A simple model of retention in ion-pair chromatography with pH variation is described. The model suggests a general procedure for method development or retention optimization in ion-pair HPLC systems. The four-solvent seven-mobile phase approach of Glajch *et al.*[6] is adapted to map sample retention as a function of pH

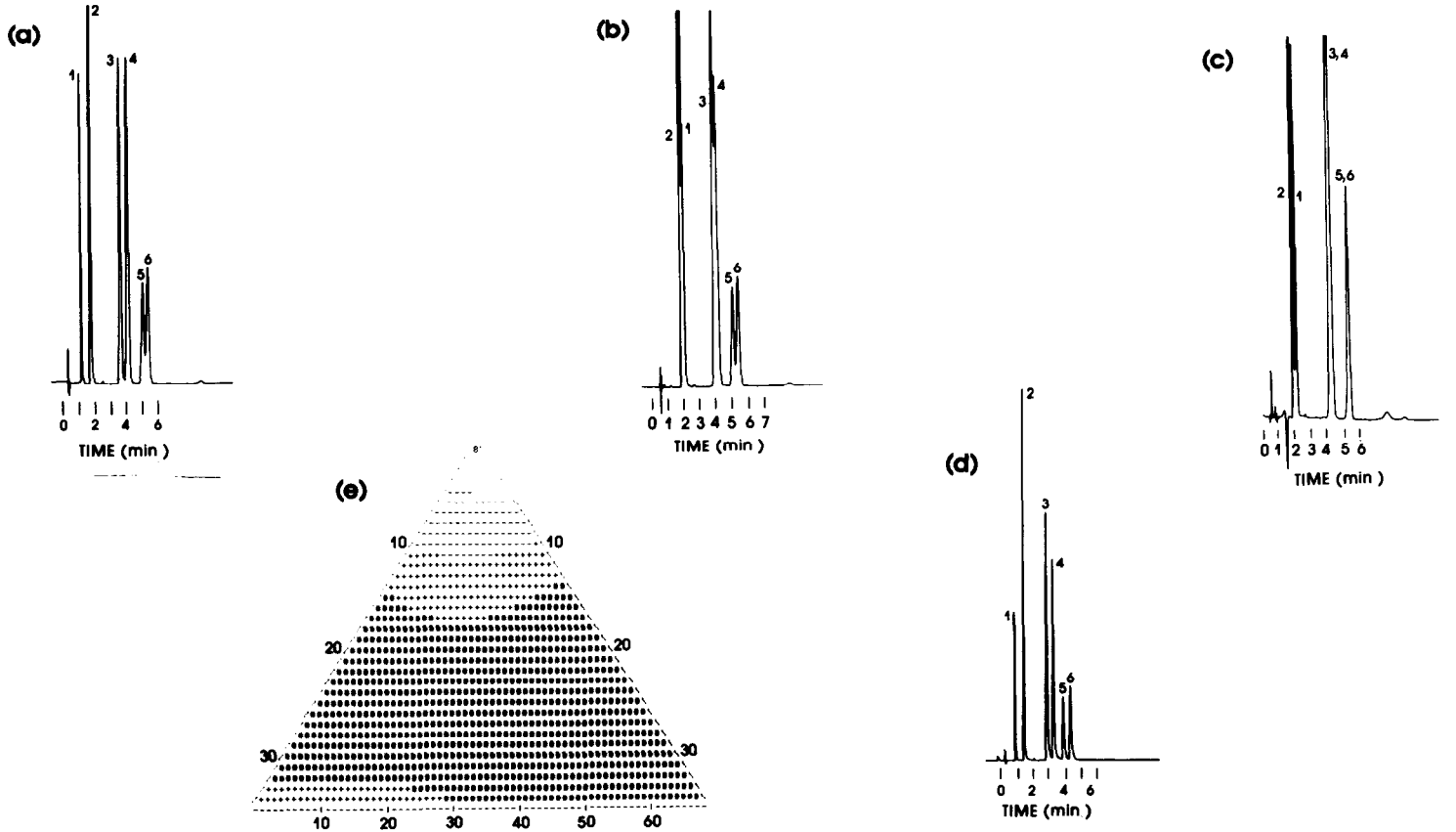

Fig. 11. Optimization of retention for six aromatic amines. 1 = *p*-Chloroaniline; 2 = 2,4-dinitroaniline; 3 = 3,4-dichloroaniline; 4 = 4-chloro-2-nitroaniline; 5 = 2,5-dichloroaniline; 6 = 2,6-dichloro-4-nitroaniline. (a) Separation with mobile phase 1 (56.9% B, 43.1% A); (b) separation with mobile phase 2 (43.7% A, 56.3% C); (c) separation with mobile phase 3 (40.9% A, 59.1% D); (d) ORM for seven mobile phases; (e) optimum separation predicted by ORM (same as mobile phase 1), with 3-μm Golden Series column replacing 6-μm Zorbax C_8. Other conditions as in Fig. 5.

(2.5–7.5) and concentration of an ion-pair reagent (0–200 m*M* hexanesulfonate). Application of this approach to a number of representative samples and the use of the DuPont Sentinel® System yielded adequate separations with a rather small number of preliminary experiments (typically about 10).

The general scheme described here for ionic or ionizable samples was previously used for both reversed-phase[6,15] and normal-phase[12] separation of non-ionized compounds. Thus most sample types can now be handled by one of these three procedures. Maximum variation in certain key variables is combined with an efficient mixture-design statistical technique to search for optimum values of these separation variables for a given sample. Difficult separations can be further optimized with other (generally less important) variables. This was illustrated earlier for the addition of column type to four-solvent optimization[44], and is shown here for addition of organic solvent type to the initial optimization via pH and ion-pairing.

SYMBOLS

k'	capacity factor of a given solute
k_0	value of k' for water as mobile phase
K_{ie}	ion-exchange equilibrium constant (eqn. 3)
K_{ie2}	value of K_{ie} for divalent solute (eqn. 10)
K_{ps}	equilibrium constant for uptake of pairing ion P^- by column packing (eqn. 1)
K_{xs}	equilibrium constant for reversed-phase retention of solute X (eqn. 4)
K'_{xs}	equilibrium constant for reversed-phase retention of solute X on a surface covered by pairing ion P^- (eqn. 12)
M^+	counter-ion for ion-pair reagent P^-; also, effective concentration of all mobile phase cations (M^+)
P^-	ion-pair reagent
ORM	overlapping-resolution map
$(pH)_{opt}$	approximate value of pH for optimum resolution of sample, as obtained in procedure of Fig. 2
(pK_a), $(pK_a)_y$	values of pK_a (eqn. 2) for solutes X and Y
s	stationary phase
S	constant in eqn. 13
t_0	retention time of an unretained compound
V_m, V_s	volumes of mobile and stationary phases within the column
X, XH^+	sample species (solute)
α	separation factor for two adjacent bands in a chromatogram (equal to ratio of their k' values)
θ_p	fraction of stationary phase surface covered by ion-pair regent P^-
θ_s	fraction of stationary phase surface not covered by P^- (equal $1 - \theta_p$)
θ_x	fraction of solute X in non-ionized form (eqn. 5)
θ_{x^+}	fraction of solute X in ionized form (XH^+) (eqn. 6)
θ_y, θ_{y^+}, $\theta_{y^{2+}}$	fraction of solute Y in forms Y, YH^+ and YH_2^{2+}, respectively

Φ volume fraction of organic solvent in mobile phase
ψ column phase ratio, equal to V_s/V_m

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