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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF BASIC DRUGS ON SILICA COLUMNS USING NON-AQUEOUS IONIC ELUENTS

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SUMMARY

The addition of ionic modifiers at low concentration to non-aqueous, primarily methanolic, eluents can facilitate the high-performance liquid chromatographic analysis of a wide range of basic compounds using microparticulate silica columns. The major factors influencing both the selectivity of the system and the retention volumes of individual analytes are the pH and the ionic strength of the eluent, although changes in the organic component of the eluent can give useful changes in selectivity, as can the use of chemically bonded stationary phases, *e.g.*, octadecylsilyl. In general, retention can be predicted to a large extent by pK_a, and even very weak bases such as benzodiazepines can be retained under strongly acidic conditions. These non-aqueous systems show high efficiency, stability and reproducibility, and give long column life. In addition, they are suitable for use with low-wavelength UV, fluorescence and electrochemical oxidation detection. The preparation of the eluent is simple, and the direct analysis of extracts performed using a non-eluting solvent is easily possible. It is clear that these systems have many advantages over bonded-phase systems using aqueous eluents in the liquid chromatographic analysis of basic drugs.

INTRODUCTION

Following the introduction of chemically bonded stationary phases for high-performance liquid chromatography (HPLC)[1], much attention has been focused on the analysis of drugs by "reversed-phase" chromatography using primarily aqueous mobile phases. Efficient performance for basic compounds is rarely obtained, however, and even when the required separation has been attained such systems have not been without difficulties in routine operation. The relatively high viscosity of water necessitates the use of comparatively high pressures when using microparticulate material in order to achieve acceptable flow-rates with the consequent risk of inducing column-sinking, although dissolution of the silica matrix even under mildly acidic

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conditions is probably a more important cause of void formation and thus deterioration in chromatographic performance[2–4]. A further problem, influencing not only practical use but also investigation of the mechanism(s) occurring in reversed-phase systems, has been the difficulty of ensuring batch-to-batch reproducibility in the production of bonded-phase material[5]. The possibility of regeneration of active sites by hydrolysis of the matrix and/or the bonded modifier must also be considered.

A major feature offered by bonded-phase systems using aqueous eluents is the ability to analyse biological fluids either directly or following removal of colloidal material such as plasma protein[6]. However, as with sample preparation prior to gas–liquid chromatography (GLC), solvent extraction at an appropriate pH provides a ready means of purification for many drugs and other lipid-soluble compounds prior to chromatographic analysis. Indeed, this can be very important in HPLC methods where detection is to be by UV monitoring at relatively low wavelengths, or where high sensitivity is required since, unless some means of sample purification is employed, the selectivity of the method is dependent largely on the resolving power of the column.

In view of these considerations, we have investigated the applicability of simple microextraction procedures, based on those developed for GLC analyses[7], to the HPLC assay of primarily thermally-labile or polar compounds where GLC analysis was inappropriate. Studies with the antiarrhythmic drug amiodarone[8] demonstrated that the addition of perchloric acid at relatively low concentrations (up to *ca.* 2 m*M*) to methanol or methanol–diethyl ether eluents was effective in promoting the elution of a variety of basic drugs from microparticulate (5 μm) silica columns as sharp, symmetrical peaks. Moreover, this system permitted the direct analysis of sample extracts and was found to be stable and reproducible, and to give long column life. In the present paper, several practical aspects of this approach to the analysis of basic drugs and other basic compounds are discussed, and some observations as to the possible mechanism of retention are presented.

EXPERIMENTAL

Materials and reagents

Methanol, hexane and methyl *tert.*-butyl ether (all HPLC grade) were obtained from Rathburn (Walkerburn, Great Britain), and chloroform, diethyl ether (both analytical-reagent grade) and diisopropyl ether (laboratory-reagent grade) from BDH (Poole, Great Britain).

Perchloric acid (60%; sp.gr. 1.54), concentrated sulphuric acid, orthophosphoric acid (sp.gr. 1.75), concentrated ammonium hydroxide solution (sp.gr. 0.88) and sodium hydroxide (all analytical-reagent grade) were obtained from BDH. Ammonium perchlorate, sodium chloride, sodium nitrate, sodium sulphate (anhydrous), potassium bromide, potassium iodide, potassium thiocyanate and potassium isothiocyanate (all analytical-reagent grade) were obtained from Hopkin and Williams (Chadwell Heath, Great Britain), sodium hexane sulphonate from Fisons (Loughborough, Great Britain), sodium lauryl sulphate from BDH and *d*-10-camphor sulphonic acid monohydrate from Aldrich (Gillingham, Great Britain).

The drugs studied were obtained from various sources, but in particular amiodarone, desethylamiodarone and the amiodarone analogues studied (see Fig. 10)

were obtained from Labaz (Brussels, Belgium). Of the internal standards used, fenethazine was obtained from Rhône-Poulenc (Paris, France), prazepam from Warner-Lambert (Eastleigh, Great Britain), benzimidazole (laboratory-reagent grade) from BDH, 5,6-benzoquinoline from Aldrich (Gillingham, Great Britain) and R17251 [N-(4-chlorophenyl)-N-(1-(3-methylbutyl)-4-piperidinyl)benzeneacetamide] from Janssen (Marlow, Great Britain).

High-performance liquid chromatography

The chromatographic systems used consisted of constant-flow reciprocating pumps (Applied Chromatography Systems, Model 750/03 or 750/04, or Waters Assoc., Model 6000A), syringe-loading sample injection valves (Rheodyne, Model 7120 or 7125, or Negretti and Zambra, Model M190) fitted with appropriate sample loops, and column effluents were monitored by UV absorption (Applied Chromatography Systems, Model 750/11, or Cecil Instruments, Model CE 212), fluorescence (Schoeffel, Model FS970) or electrochemical oxidation[9]. Stainless-steel tubing (0.25 mm I.D.) was used to connect the outlet port of the injection valve to the analytical column, a stainless steel tube (125 or 250 × 4.9 mm I.D.). The column-packing materials used were Spherisorb S5W silica, S5 ODS1, S5 Phenyl and S5 Nitrile (Phase Separations), Zorbax TMS (DuPont) (all obtained ready-packed from Hichrom, Woodley, Great Britain), Ultrasphere 5 μm ODS (Altex Scientific, obtained from Anachem, Luton, Great Britain), and Syloid 74 silica (W. R. Grace, St. Neots, Great Britain), graded by aqueous sedimentation to give a mean particle size of 5 μm and heated at 800°C for 24 h before packing from a methanol slurry. The analyses were performed at ambient temperature (normally 22°C) and at a flow-rate of (normally) 2 ml/min, maintained by a pressure of *ca.* 50 bar (700 p.s.i.) for a 125-mm column packed with Spherisorb 5 Silica using methanol as eluent.

RESULTS AND DISCUSSION

The use of non-aqueous eluents in the analysis of basic drugs by HPLC using UV absorbance detection has been restricted not only by the relatively high UV cut-off of many commonly used solvents such as dichloromethane, but also by the risk of deactivation of silica columns by adsorption of water. On the other hand, it was felt that where solvent extraction was to be used as the method of sample preparation, the mobile phase chosen should permit the direct analysis of the resulting extract. This approach was adopted by Lagerström and Persson[10] for the analysis of a number of antiarrhythmic drugs using silica columns, although, in the main, chlorinated solvents modified by the addition of aqueous perchloric acid (1 *M*) and methanol were used. Westerlund *et al.*[11] again used dichloromethane modified with aqueous perchlorate and in this case butanol in the analysis of zimelidine and some analogues. Characteristics of this type of system included the elution of secondary amines such as norzimelidine and N-desalkyldisopyramide after the parent compounds.

Other workers have also used silica columns but with methanol–aqueous buffer eluents at alkaline pH[12] or similar eluents containing cetyltrimethylammonium bromide and used in the pH range 5–9[13,14]. These latter systems were characterised by the elution order primary (1°) < secondary (2°) < tertiary (3°) < quaternary (4°) for amine analogues of the 3° amine imipramine at pH 7.7, *i.e.* a similar order to that

expected in bonded-phase systems with analogous aqueous eluents. Crommen[15] and Svendsen and Greibrokk[16] again observed a "reversed-phase" elution order for a variety of primarily basic organic compounds using silica columns but with aqueous solutions of organic and inorganic acids and acid salts as eluents. Some separations were also characterised by the poor efficiencies attained. An alternative approach to the HPLC of polar organic compounds using silica has been suggested by Schwarzenbach[17], who pre-coated the column with acidic or basic salts and used non-aqueous eluents such as hexane–diethyl ether. However, use of more polar mobile phases was found to shorten the life of the column considerably. Thus, although Lagerström and Persson[10] reported that the direct injection of dichloromethane extracts was possible when using a methanol–aqueous buffer eluent, it was felt prudent to investigate the use of alternative mobile phases.

In our initial studies it was found that methanol or methanol–diethyl ether would not elute amiodarone from a 125-mm column packed with Spherisorb 5 silica.

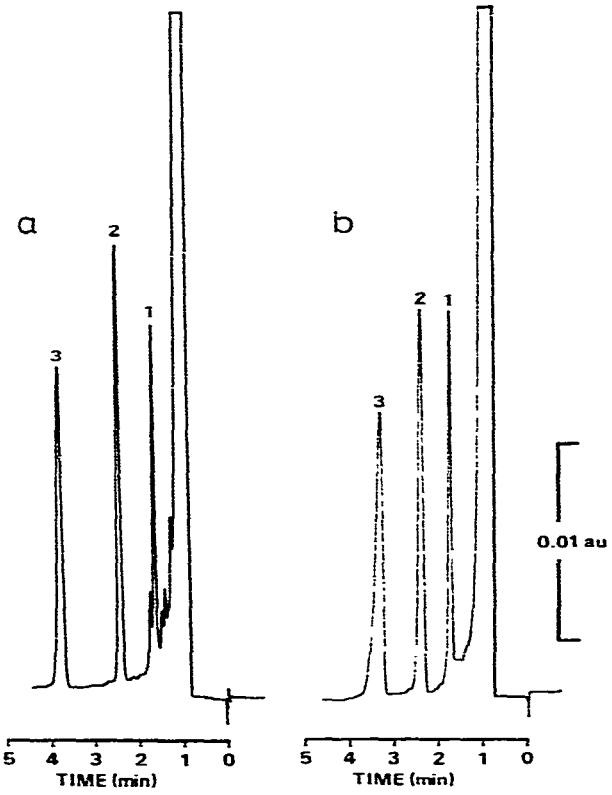


Fig. 1. Chromatography of desethylamiodarone (1), amiodarone (2) and fenethazine (internal standard) (3) on a 125-mm column packed with Spherisorb 5 silica. Flow-rate: 2.0 ml/min. Detection: UV, 240 nm. Injection: 100 μl of diisopropyl ether extract of plasma from an amiodarone-treated patient (*cf.* ref. 8). (a) "New" column. Eluent: methanol–diethyl ether (85:15) containing perchloric acid (0.02%, v/v; 1.85 m*M*). Plasma amiodarone concentration, 1.1 mg/l. (b) Column after over 2-year routine use. Eluent: Methanol–diethyl ether (85:15) containing perchloric acid (0.01%, v/v; 0.93 m*M*). Plasma desethylamiodarone and amiodarone concentrations, 1.3 and 1.1 mg/l, respectively.

However, the addition of perchloric acid at low concentration (typically 0.02%, v/v; 1.85 m*M*) to the eluent had a dramatic effect: amiodarone was now eluted within 3 min as a sharp, symmetrical peak (Fig. 1). The retention time of amiodarone was found to be dependent primarily on the perchloric acid concentration in the eluent, and the direct analysis of diisopropyl ether extracts of plasma proved easily possible using this system[8]. Greving *et al.*[18] have also described the analysis of some tricyclic antidepressants and phenothiazines using methanolic solutions of sodium bromide or perchlorate on silica columns. Subsequent studies have shown that these systems have many similarities to those using methanol–aqueous ammoniacal eluents with silica columns for the analysis of basic drugs[12,19], and are applicable to a wide range of basic compounds.

Characteristic features of this type of system are (i) basic compounds only are retained under conditions where they are appreciably ionised, (ii) increases in the ionic strength of the eluent produce decreases in retention and (iii) for a range of structurally similar compounds, those substances of apparently greater polarity are often eluted *before* the remaining compounds. The separations attained are thus similar in many respects to those expected for "reversed-phase" systems using aqueous eluents, and this is illustrated in Figs. 1 and 2 where the 2° amine metabolites desethylamiodarone and nordextropropoxyphene elute before the parent 3° amines in each case. Hydroxylated analogues or metabolites may be eluted even more rapidly than demethylated or dealkylated analogues, and this is illustrated in Fig. 3, where the elution order for diazepam and its three principal plasma metabolites is temazepam (3-hydroxydiazepam), oxazepam (3-hydroxynordiazepam), nordiazepam and finally diazepam.

Retention/selectivity adjustment using non-aqueous ionic eluents

The use of non-aqueous ionic eluents has many advantages in the analysis of basic compounds by HPLC using silica columns. Of paramount importance is the great flexibility offered by the ability to control retention and/or selectivity by adjustment of (i) the pH of the eluent, (ii) the ionic strength, (iii) the composition of the organic component of the eluent and (iv) by the use of bonded-phase materials.

Influence of eluent pH on retention. Using these non-aqueous ionic eluent systems, most basic organic substances can be retained, but only under conditions where they are appreciably ionised. Indeed, retention can be predicted to a large extent from the pK_a of the analyte.

The variation in retention with eluent pH for a test mixture of 1°, 2° and 3° amines on a silica column is shown in Fig. 4. The pH measurements were performed using a standard glass pH electrode immersed in the eluent and were not corrected for the absence of water from this medium. It is clear that maximum retention is obtained at intermediate pH values. The decreased retention at relatively low pH is probably due to suppression of the ionisation of weakly acidic silanol groups on the silica surface, whereas at high pH the ionisation of the analyte is suppressed and this again leads to decreased retention. Even very weak bases such as the benzodiazepines can be retained (maximally) under strongly acidic conditions (Fig. 3), higher eluent pH values giving decreased retention.

In general, optimum selectivity towards a given analyte is attained by the use of as high an eluent pH as possible since the retention of weaker bases will be minimized.

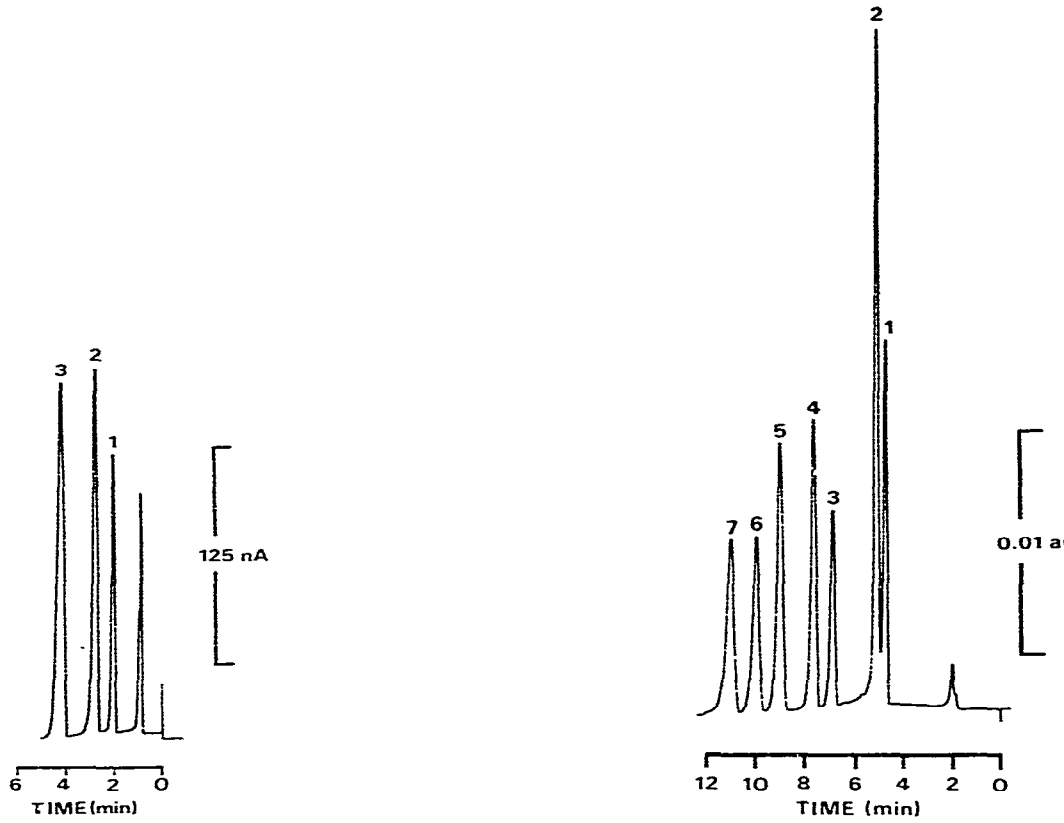


Fig. 2. Chromatography of nordextropropoxyphene (1), dextropropoxyphene (2) and amitriptyline (3) on a 125-mm column packed with Spherisorb 5 silica. Eluent: methanol containing ammonium perchlorate (10 m*M*) adjusted to apparent pH 6.7 by addition of 1 ml/l methanolic sodium hydroxide (0.1 *M*). Flow-rate: 2.0 ml/min. Detection: electrochemical oxidation using V25 glassy carbon electrode, 1.2 V applied against Ag/AgCl reference electrode. Injection: 20 µl of methanolic solution containing 2 mg/l of each compound.

Fig. 3. Chromatography of temazepam (1), oxazepam (2), nitrazepam (3), desalkylflurazepam (4), nordiazepam (5), prazepam (6) and diazepam (7) on a 250-mm column packed with Spher_orb 5 silica. Eluent: methanol containing perchloric acid (0.02%, v/v; 1.85 m*M*). Flow-rate: 2.0 ml/min. Detection: UV, 240 nm. Injection: 100 µl of methanolic solution containing 1.0 mg/l of each compound.

On the other hand, the use of a strongly acidic eluent is indicated if a qualitative analysis is to be performed since the number of compounds retained will be maximized. Change of eluent pH can be used to achieve resolution of compounds with different pK_a values (Fig. 5), although where the pK_a values of co-eluting analytes are the same, changes in eluent pH are unlikely to achieve additional resolution.

A further consideration in the choice of eluent pH is that some 3° amines such as amiodarone show higher efficiencies under strongly acidic conditions using methanolic eluents (Fig. 5), while some complex bases such as flurazepam give rise to tailing peaks under conditions where more than one basic group should be ionised (Fig. 6). It is obviously desirable to perform an analysis under conditions of maximal

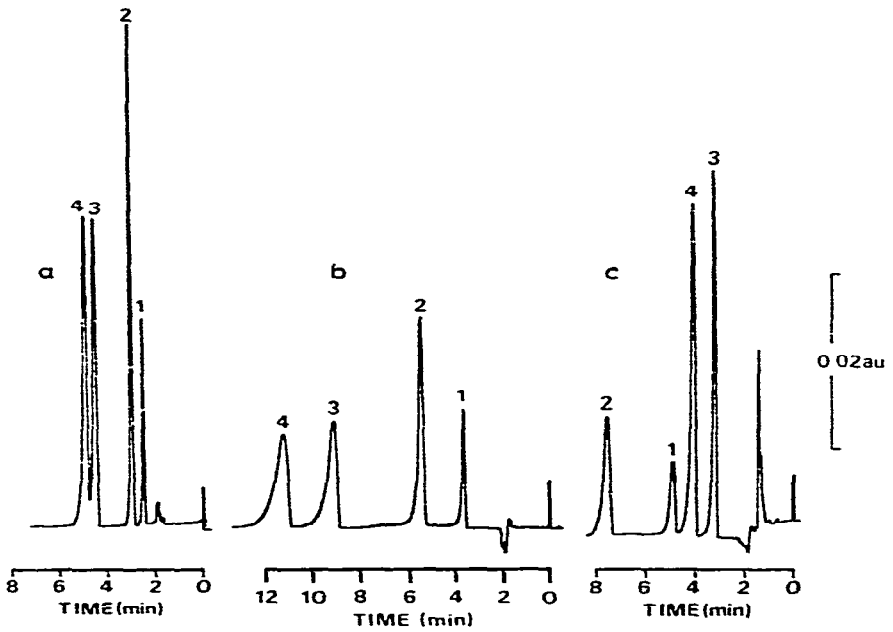


Fig. 4. Influence of eluent pH on the chromatography of amphetamine (pK_a 9.9) (1), nortriptyline (pK_a 10.0) (2), amitriptyline (pK_a 9.4) (3) and imipramine (pK_a 9.5) (4) on a 230-mm column packed with Syloid 74 silica (800°C baked). Flow-rate: 2.0 ml/min. Detection: UV, 254 nm. Injection: 25 µl of methanolic solution containing amphetamine (100 mg/l) and the remaining compounds (all 10 mg/l). Eluent: methanol containing ammonium perchlorate (10 m*M*). (a) Eluent adjusted apparent pH 1.0 by addition of 5 ml/l methanolic perchloric acid (0.1 *M*). (b) Eluent adjusted to apparent pH 6.7 by addition of 1 ml/l methanolic sodium hydroxide (0.1 *M*). (c) Eluent adjusted to apparent pH 9.6 by addition of 180 ml methanolic sodium hydroxide (10 m*M*) to 220 ml eluent.

efficiency (other factors being equal), especially if quantitative work is to be performed and/or high sensitivity is required, and the conditions required to give optimal efficiencies for both of the above compounds using a methanolic eluent have been ascertained (Figs. 5 and 6). The reason why these compounds should show different efficiencies under different pH conditions is not clear, although reduced solubility in the eluent, thus giving rise to tailing peaks, is one possibility. This could also explain the observation that quaternary ammonium compounds such as thiazinium sulphoxide[18] or emepronium give rise to tailing peaks using methanol–perchloric acid or methanol–ammonium or sodium perchlorate as eluents.

Effect of eluent ionic strength. In each case where these non-aqueous ionic eluent systems have been used, the ionic strength required to promote elution of the analytes studied at a practical capacity ratio (k') of 2–6 has been between 0.5 and 20 m*M*. Higher ionic strengths give rise to shorter retention times[18] and *vice versa*, although lower concentrations of perchloric acid than 0.5 m*M* permitted peak tailing with many analytes and primarily methanolic eluents. The effect of ionic strength on retention is clearly interrelated with eluent pH in that at very low pH a lower ionic strength is required to promote the elution of a given analyte at a given retention volume than at higher pH values (Fig. 5).

Greving *et al.*[18] reported that different selectivity could be obtained on silica

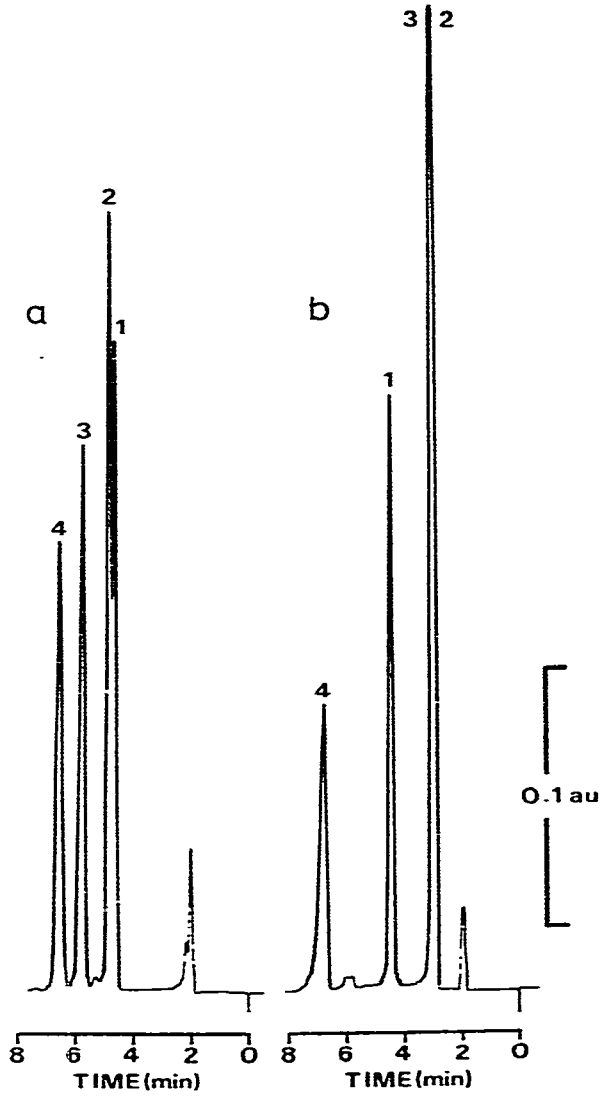


Fig. 5. Influence of eluent pH on the chromatography of desethylamiodarone (1) nordiazepam (2), diazepam (3) and amiodarone (4) on a 250-mm column packed with Spherisorb 5 silica. Flow-rate: 2.0 ml/min. Detection: UV, 240 nm. Injection: 50 μl of methanolic solution of desethylamiodarone and amiodarone (both 30 mg/l), and nordiazepam and diazepam (both 15 mg/l). (a) Eluent: methanol containing perchloric acid (0.02%, v/v; 1.85 m*M*), apparent pH < 0. (b) Eluent: methanol containing perchloric acid (0.10%, v/v; 9.25 m*M*), adjusted to apparent pH 4.0 with 92.6 ml/l methanolic sodium hydroxide (0.1 *M*).

columns using sodium bromide or sodium perchlorate in methanol as eluents, and this was attributed to differences in ion-pair formation. However, no account was taken of possible differences in pH between these eluents. Although we have not investigated systematically the effect of variations in the nature of the cations or anions used on retention or selectivity, we have not observed any major effects attrib-

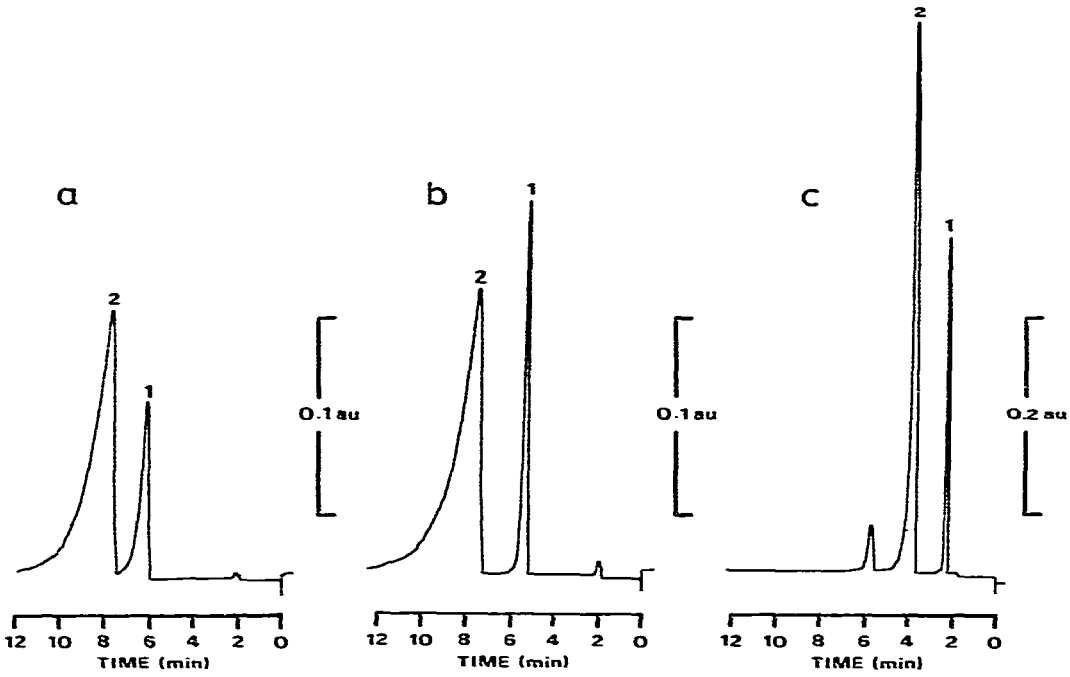


Fig. 6. Influence of eluent pH on the chromatography of flurazepam (1) and quinine (2) on a 250-mm column packed with Spherisorb 5 silica. Flow-rate: 2.0 ml/min. Detection: UV, 240 nm. Injection: 100 μl of methanolic solution of both flurazepam (20 mg/l) and quinine (100 mg/l). (a) Eluent: methanol containing perchloric acid (0.10%, v/v; 9.25 m*M*). apparent pH < 0. (b) Eluent: methanol containing ammonium perchlorate (10 m*M*), adjusted to apparent pH 6.7 by addition of 1.0 ml/l methanolic sodium hydroxide (0.1 *M*). (c) Eluent: methanol containing ammonium perchlorate (32.8 m*M*) adjusted to apparent pH 9.2 by addition of conc. ammonia solution (3.55 ml perchloric acid + 9.2 ml conc. ammonia solution per litre of eluent).

utable to the use of different ionic modifiers at constant pH values. Ammonium salts such as ammonium perchlorate are useful modifiers since they are usually adequately soluble in methanol and can act as buffers at relatively high eluent pH, but some potassium or sodium salts such as chloride, bromide or nitrate may also be used. The addition of methanolic sodium hydroxide (0.1 *M*) provides a convenient mode of adjusting eluent pH at relatively high pH values. For low pH eluents, perchloric acid, which is highly ionised in organic media, used alone without any additional cations has proved a satisfactory modifier. Analogous results have been obtained using sulphuric acid in place of perchloric acid, although weak acids such as orthophosphoric acid were found not to be effective. The use of other inorganic salts such as sodium sulphate is restricted by their poor solubility in methanol, while other ions such as iodide, thiocyanate and isothiocyanate have high UV absorption even at the low concentrations required for this work. Indeed, the use of bromide is restricted by the UV cut-off of this ion at approximately 220 nm.

Greving *et al.*[18], using LiChrosorb or LiChrosfer silica columns, reported that organic anions in methanolic solution were (i) more likely to dominate chromatographic behaviour and (ii) gave rise to interferences due to interactions with the

stationary phase when compared to inorganic ions such as bromide or perchlorate. In contrast, we have found that compounds such as sodium hexane sulphonate, sodium lauryl sulphate and camphor sulphonic acid, when used at pH values and concentrations similar to those of inorganic modifiers such as perchloric acid, gave rise to broadly similar retention and selectivity for the compounds studied, and did not give rise to extraneous, interfering peaks. An example of the use of an organic ion is the analysis of propranolol and 4-hydroxypropranolol on a silica column using camphor sulphonic acid (1 m*M*) in methanol as eluent (Fig. 7). It is possible that the use of organic anions may give rise to useful changes in selectivity when compared to inorganic anions, especially when using bonded-phase materials (see below). Indeed, such an effect has been suggested by some observations made using silica columns, but further work is needed to clarify this point.

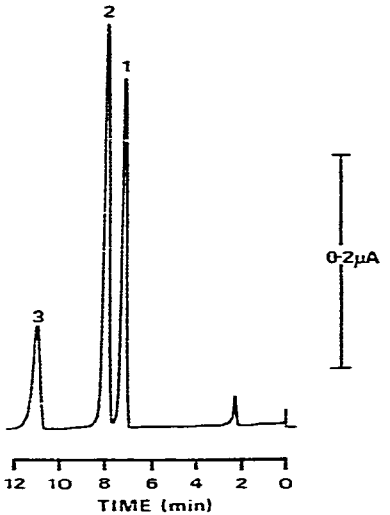


Fig. 7. Chromatography of 4-hydroxypropranolol (1), propranolol (2) and benzimidazole (internal standard) (3) on a 250-mm column packed with Spherisorb 5 silica. Eluent: methanol containing camphor sulphonic acid (1 m*M*). Flow-rate: 2.0 ml/min. Detection: fluorescence, excitation 215 nm, no emission filter. Injection: 70 μl of methanolic solution containing 4-hydroxypropranolol and propranolol (both 0.5 mg/l) and benzimidazole (3 mg/l).

Changes in eluent solvent composition. A further parameter which can be used to adjust selectivity is the solvent component of the eluent, and this is well illustrated by the analysis of codeine and morphine. These components are not resolved on a silica column using methanol containing ammonium perchlorate (13.1 m*M*) adjusted to apparent pH 9.2 as eluent. However, it was found that the use of methanol–chloroform (40:60) as eluent solvent gave a good separation of these components (Fig. 8). Changes in eluent solvent composition may prove especially useful in adjusting selectivity when using bonded stationary phases (see below).

The effect of the addition of water to the eluent has been investigated in view of similarities between the elution order of amines (1° < 2° < 3° < 4°) obtained on these non-aqueous ionic eluent systems to the elution order of such compounds on silica columns using primarily aqueous eluents[13–16]. In the event, the addition of

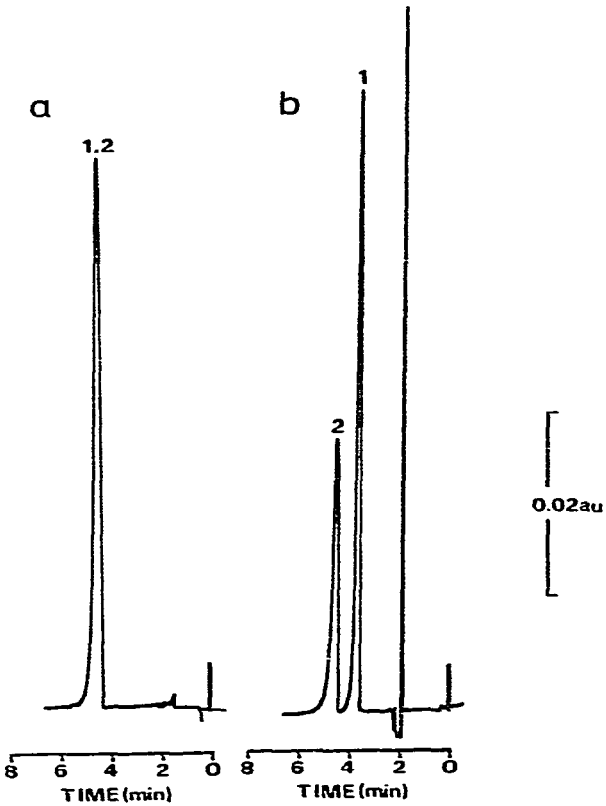


Fig. 8. Chromatography of codeine (1) and morphine (2) on a 250-mm column packed with Syloid 74 silica (800°C baked). Flow-rate: 2.0 ml/min. Detection: UV, 254 nm. Injection: 5 μl of methanolic solution containing 1 g/l of each compound. Eluent: ammonium perchlorate (13.1 m*M*) adjusted to apparent pH 9.2 by addition of conc. ammonia solution (1.42 ml perchloric acid + 3.68 ml conc. ammonia solution per litre of eluent). (a) Eluent solvent: methanol. (b) Eluent solvent: methanol–chloroform (40:60).

water (10% or 20%, v/v) to a methanolic eluent had no major effect on the chromatography of a mixture of 1°, 2° and 3° amines when both the pH and the ionic strength of the eluent were held constant (Fig. 9), although the pressure required to maintain a given flow-rate using the methanol–aqueous mixture was considerably higher than that required when using methanol alone.

Use of chemically bonded stationary phases. Although great flexibility of approach is offered by the ability to vary the pH, the ionic strength and the composition of the organic component of the eluent when using silica columns, the availability of a variety of chemically bonded stationary phases offers a further means of influencing the selectivity of the system. An example of the use of an octadecylsilyl-bonded (ODS) phase in this mode, the analysis of amiodarone and eight analogues (Fig. 10) on both Spherisorb 5 silica and ODS1, is illustrated in Fig. 11. The addition of perchloric acid was required to promote the elution of the compounds under study from the ODS column, and analogous concentrations gave rise to similar retention times on both systems. Since Spherisorb 5 ODS1 is relatively lightly loaded (total carbon *ca.* 7%, w/w) and not end-capped (data from Phase Separations), it is clear

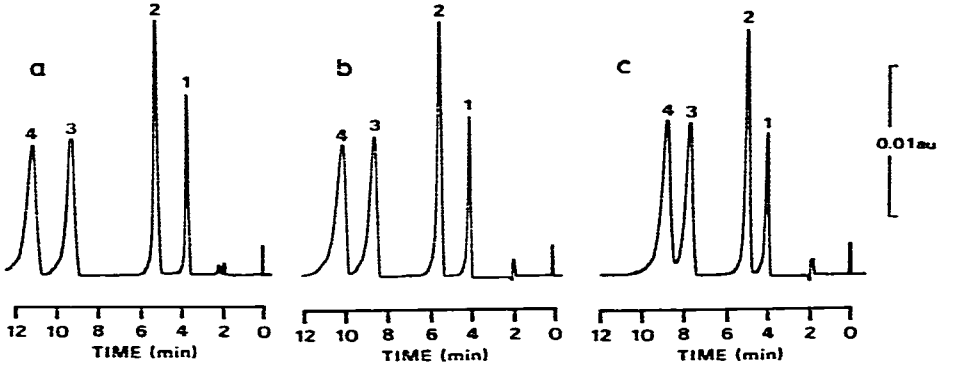
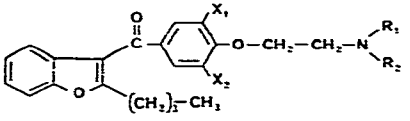


Fig. 9. Influence of water on the chromatography of amphetamine (1), nortriptyline (2), amitriptyline (3) and imipramine (4) on a 230-mm column packed with Syloid 74 silica (800°C baked). Flow-rate: 2.0 ml/min. Detection: UV, 254 nm. Injection: 25 μl of methanolic solution containing amphetamine (100 mg/l) and the remaining compounds (all 10 mg/l). Eluent: ammonium perchlorate (10 m*M*) adjusted to apparent pH 6.7 by addition of 1.0 ml/l methanolic sodium hydroxide (0.1 *M*). (a) Eluent solvent: methanol, *Dp* (pressure drop) = 143 bar (2000 p.s.i.). (b) Eluent solvent: methanol–water (90:10), *Dp* = 214 bar (3000 p.s.i.). (c) Eluent solvent: methanol–water (80:20), *Dp* = 271 bar (3800 p.s.i.).

that the dominant influence in the separations attained was probably interaction with surface silanols in both instances. Indeed, other non end-capped Spherisorb materials, *e.g.* phenyl and cyanopropyl[8], show similar overall efficiencies and retention characteristics to unmodified silica.

In order to investigate the use of bonded-phase material which had clearly been extensively silanized, columns packed with (i) Zorbax TMS (DuPont) (trimethylsilyl-treated) and (ii) Ultrasphere ODS (Altex) (octadecylsilyl- and trimethylsilyl-treated)



Analogue No	R_1	R_2	X_1	X_2
1	C_2H_5	C_2H_5	I	I
2	C_2H_5	H	I	I
3	H	H	I	I
4	C_2H_5	C_2H_5	I	H
5	C_2H_5	H	I	H
6	H	H	I	H
7	C_2H_5	C_2H_5	H	H
8	C_2H_5	H	H	H
9	H	H	H	H

Fig. 10. Structural formulae of amiodarone, desethylamiodarone and the seven amiodarone analogues studied (*cf.* Fig. 11).

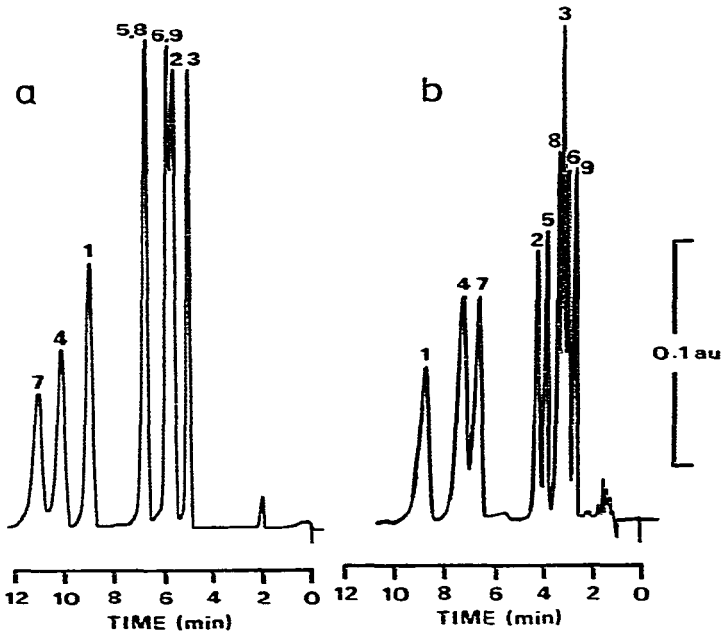


Fig. 11. Chromatography of amiodarone, desethylamiodarone and seven amiodarone analogues (Fig. 10) on a 250-mm column. Eluent: methanol containing perchloric acid (0.03%, v/v; 2.78 m*M*). Flow-rate: 2.0 ml/min. Detection: UV, 240 nm. Injection: 100 μl of methanolic solution (20 mg/l) of each compound. (a) Column packed with Spherisorb 5 silica. (b) Column packed with Spherisorb 5 ODS1.

were studied. The addition of perchloric acid to the eluent (methanol) was necessary in both cases to promote the elution of the compounds studied (amiodarone and desethylamiodarone). Again, in both cases, the retention time was dependent on the ionic strength and desethylamiodarone was eluted before the parent compound (Fig. 12). However, the peak shape obtained on both columns at similar retention times was very poor (markedly tailing peaks), although when used conventionally with aqueous eluents both columns gave rise to sharp, symmetrical peaks for the analytes studied (primarily benzodiazepines and acidic compounds).

Practical considerations in the use of non-aqueous ionic eluents

In addition to the great flexibility offered by the ability to adjust retention and selectivity as discussed in the previous paragraphs, the use of non-aqueous ionic eluents has many practical advantages in the HPLC analysis of basic drugs. These considerations apply especially in (i) the preparation and use of eluents, (ii) choice of injection volume and conditions and (iii) column stability and reproducibility. Before consideration of these factors, however, it must be emphasised that extra-column effects can exert a marked deleterious influence on the chromatograms obtained with a given system. In order to minimise such effects, stainless-steel tubing (0.25 mm I.D.) was used to connect the outlet port of the injection valve to the analytical column and the analytical column to the detector using zero dead-volume fittings, and the detector time-constants employed were the lowest attainable with the instruments used[20].

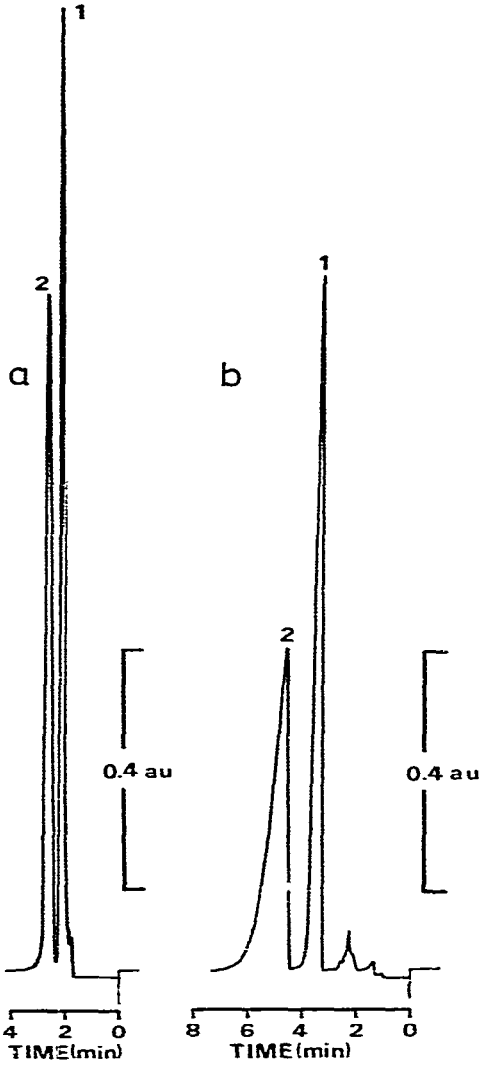


Fig. 12. Chromatography of desethylamiodarone (1) and amiodarone (2) on a 250-mm column. Eluent: methanol containing perchloric acid (0.01%, v/v; 0.93 m*M*). Flow-rate: 2.0 ml/min. Detection: UV, 240 nm. Injection: 15 µl of methanolic solution (1.0 g/l) of both compounds. (a) Column packed with Zorbax TMS. (b) Column packed with 5 µm Ultrasphere ODS.

It was also ensured that the response time of the recorders used was appropriately rapid.

Preparation and use of eluents. We have found that, where required, the addition of 60% perchloric acid or concentrated sulphuric acid to the organic component of the eluent in the small quantities required (100 µl to 500 ml will give 0.02%, v/v) to be a safe and convenient method of preparing mobile phases. Flushing of the chromatograph with pure solvent after use was only necessary with eluents containing salts such as sodium bromide, although sonication and subsequent filtration of

the solutions of such compounds prior to use[18] was not required given the very low concentrations employed in this work. Helium degassing and precautions to prevent the back-diffusion of oxygen into the mobile phase were only found to be necessary when working at high sensitivity with UV absorption at 215 nm[21]. No detrimental effects to either the column packing or the chromatograph itself were observed with any of eluents used, including acidic solutions of chloride or bromide.

Further considerations are that methanol is a relatively cheap HPLC-grade solvent and has low inhalational toxicity. The collection of effluent fractions is simple and change of eluents is easily accomplished with the exception of changes from low (perchloric acid eluent) to neutral pH values. This is probably due to the buffering effect of silica at these pH values, and such changes are best accomplished by purging the columns with several column volumes of an eluent of relatively high ionic strength before reverting to the use of the normal eluent.

Choice of injection volume and conditions. Although relatively selective, UV detection is inherently less sensitive for many compounds when compared, to, for example, flame-ionization detection in GLC. However, the amount of substance injected may often be increased to compensate for this if sufficient sample is available. Indeed, it was found that the injection of up to 2000 µl of a solution of analytes in methyl *tert.*-butyl ether did not give rise to noticeable peak broadening or tailing (*cf.* Figs. 13 and 14). This is probably due to the absorption of the analyte(s) onto the top of the analytical column, followed by desorption as the high-ionic-strength mobile phase displaces the extraction solvent, *i.e.* is analogous to the use of a "non-eluting" solvent to achieve sample pre-concentration in "reversed-phase" chromatography

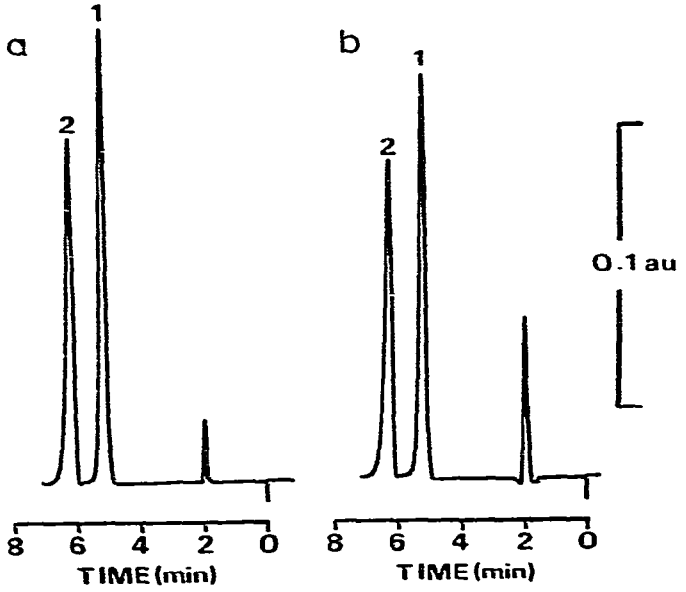


Fig. 13. Chromatography of nordiazepam (1) and diazepam (2) on a 250-mm column packed with Spherisorb 5 silica. Eluent: methanol containing perchloric acid (0.02%, v/v; 1.85 m*M*). Flow-rate: 2.0 ml/min. Detection: UV, 240 nm. Injection: 100 µl solvent containing 1 µg of each compound. (a) Injection solvent: eluent. (b) Injection solvent: methyl *tert.*-butyl ether.

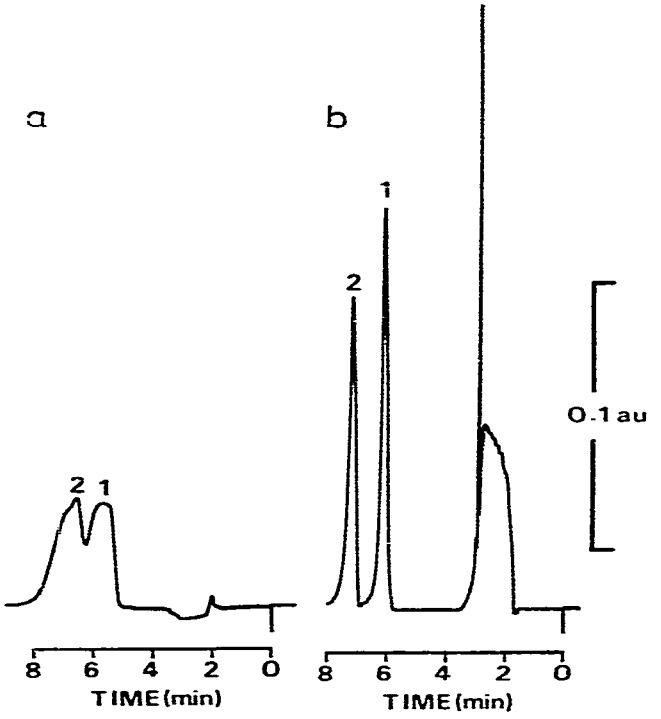


Fig. 14. As Fig. 13 except for injection volume (2000 μl).

with aqueous eluents or in "normal-phase" chromatography[22]. Further evidence for this view is provided by the observation that large-volume injections performed using a solution of analytes prepared in column eluent were accompanied by a marked decrease in efficiency (Fig. 14).

Methyl *tert.*-butyl ether has proved to be a useful extraction–injection solvent. The use of this compound was prompted by observations[23] as to the advantages of this compound as a chromatographic eluent. These same properties, *i.e.* lower volatility than diethyl ether and greatly enhanced stability (no stabilizing agent is required) when compared to both diethyl and diisopropyl ethers, are also advantageous in an extraction solvent, and this is especially important in HPLC where the column effluent is to be monitored at relatively low UV wavelengths. Methyl *tert.*-butyl ether is miscible with methanol and hexane in all proportions, and thus can be used for direct injection onto chromatographic systems where these solvents are used as eluents. Methyl *tert.*-butyl ether shares with other ethers the advantage of having a lower specific gravity than water, and this enables most of the extract of an aqueous solution to be removed without risk of contamination and without prior removal of the aqueous phase.

Column stability and reproducibility. Silica is a relatively cheap column packing material, and it is easy to pack columns of high efficiency. In common with Greving *et al.*[18], we have found these non-aqueous ionic eluent systems to be stable and reproducible, and to give long column life. This latter consideration is especially important, and contributory factors probably include the relatively low pressures needed, the

absence of all but trace amounts of water, hence minimizing problems attributable to the dissolution of the column packing, and the relatively high purity of solvent extracts, especially when compared to the results of protein precipitation methods. Such systems, used without a pre-column or in-line filter at flow-rates of the order of 2 ml/min, have been operated routinely in conjunction with simple solvent-extraction procedures for up to 2 years (Fig. 1b), although it should be noted that the eluent ionic strength required to maintain a given elution volume for a particular analyte was often decreased after prolonged use.

Any deterioration in efficiency or peak shape which was observed was often attributable to contamination of the chromatographic system, accumulation of insoluble material at the top of the column or in the microbore tubing from the injection port, or to the formation of small voids in the column packing after prolonged use. Flushing with diethyl ether–hexane (*ca.* 50 ml) and/or a relatively high ionic strength eluent (again *ca.* 50 ml) was often successful in removing soluble contaminants from the columns. Simple cleaning and, if necessary, repacking of the top 2–3 mm of the column using a slurry of the appropriate packing material prepared in methanol (silica) or chloroform (bonded phases) was often effective in prolonging the life of the column. Slurry packing is made easier by the fact that the eluent and slurrying solvent are both organic.

The occurrence of voids in the packing of the analytical column was an occasional observation, even when using a pre-column (100 × 2 mm I.D.) packed with LiChroprep 15–25 μm silica (E. Merck). The use of a silica guard-column in line between the pump and the inlet port of the injection valve may serve to further prolong column life, as advocated for aqueous bonded-phase systems[3], and re-cycling of the eluent may also prove practicable.

Implications for qualitative and quantitative analysis

It is clear from the results presented in Table I that most basic drugs can be retained on a silica column under strongly acidic conditions, and can be eluted on a practically useful time-scale. Exceptions to this rule are some benzodiazepines such as lorazepam and clobazam which are only poorly retained using an eluent with a high methanol content, and some complex bases such as flurazepam and quinine which give rise to tailing peaks at low eluent pH (Fig. 6). Quaternary ammonium compounds also give rise to tailing peaks under most of the eluent conditions studied. Nevertheless, taken together with the ability to monitor the column effluent at relatively low UV wavelengths, *e.g.* 210 nm, it is clear that these systems may prove useful in qualitative analyses.

Acidic/neutral drugs are for practical purposes not retained (Table I; see also ref. 8) and therefore can be eliminated as potential sources of interference. Further information as to compound identity may be obtained by observation of any changes in analyte retention with eluent pH, in addition to data which may be obtained from either stopped-flow UV scanning or multiple-wavelength monitoring, or indeed the use of additional detection systems. It should be emphasised that the elution order for relatively polar compounds such as N-dealkylated metabolites differs from that expected from conventional adsorption chromatography systems[18].

The applicability of these non-aqueous ionic eluent systems to quantitative analyses using UV and fluorescence detection has been demonstrated[8,24]. Such sys-

TABLE I

RETENTION DATA OF THE COMPOUNDS STUDIED ON A 250-mm COLUMN PACKED WITH SPHERISORB 5 SILICA USING METHANOL–HEXANE (85:15) CONTAINING PERCHLORIC ACID AS ELUENT

Detection: UV, 215 nm. Flow-rate: 2.0 ml/min.

Perchloric acid concentration	*Compound*	*Relative retention time*	*k'**
0.02% (v/v) (1.85 m*M*)**	Amylobarbitone	0.22	0.27
	Phenobarbitone	0.22	0.27
	Clobazam	0.24	0.38
	Caffeine	0.25	0.44
	Camazepam	0.26	0.50
	Demoxepam	0.26	0.50
	Carbamazepine	0.27	0.55
	Flunitrazepam	0.27	0.55
	Theophylline	0.27	0.55
	Carbamazepine-10,11-epoxide	0.28	0.61
	Lorazepam	0.28	0.61
	Clonazepam	0.35	1.01
	Desmethylflunitrazepam	0.45	1.59
	Temazepam	0.48	1.76
	Oxazepam	0.51	1.93
	Azapropazone	0.56	2.22
	Labetalol	0.60	2.45
	Tocainide	0.60	2.45
	4-Hydroxypropranolol	0.63	2.62
	Mexiletine	0.63	2.62
	Amphetamine	0.65	2.74
	Ajmaline	0.68	2.91
	Sotalol	0.68	2.91
	Propranolol	0.69	2.97
	Nitrazepam	0.70	3.03
	Metoprolol	0.71	3.08
	Oxprenolol	0.71	3.08
	Flurazepam N_1-ethanol	0.72	3.14
	Nordextropropoxyphene	0.74	3.26
	7-Acetamidonitrazepam	0.75	3.31
	Desalkylflurazepam	0.78	3.49
	Atenolol	0.79	3.54
	D620 (verapamil metabolite[24])	0.79	3.54
	Desethylamiodarone	0.81	3.66
	Norverapamil	0.86	3.95
	Methylamphetamine	0.86	3.95
	Maprotiline	0.89	4.12
	Nordiazepam	0.89	4.12
	Pinazepam	0.92	4.29
	Alinidine	0.94	4.41
	Nordoxepin	0.96	4.52
	Benzimidazole (Internal standard)	0.98	4.64
	Prazepam	1.00 (= 9.20 min)	4.75
	Chlordiazepoxide	1.01	4.81

TABLE I *(continued)*

Perchloric acid concentration	*Compound*	*Relative retention time*	*k'**
	Dextropropoxyphene	1.07	5.15
	Lignocaine	1.07	5.15
	D617 (Verapamil metabolite[24])	1.10	5.33
	Diazepam	1.13	5.50
	Amiodarone	1.32	6.59
	Verapamil	1.51	7.68
0.05% (v/v)(4.63 m*M*)**	Lorazepam	0.47	0.40
	Oxazepam	0.67	0.99
	Desethylamiodarone	0.77	1.29
	7-Acetamidonitrazepam	0.78	1.32
	Desalkylflurazepam	0.82	1.44
	Nortrimipramine	0.82	1.44
	Norclomipramine	0.84	1.50
	Nortriptyline	0.86	1.56
	Desipramine	0.87	1.59
	Nordiazepam	0.88	1.62
	Maprotilene	0.89	1.65
	Norpethidine	0.90	1.68
	Protriptyline	0.90	1.68
	Prazepam	1.00 (= 4.76 min)	1.98
	Diazepam	1.09	2.24
	Lorcainide	1.10	2.27
	Methaqualone	1.12	2.33
	Amiodarone	1.15	2.42
	Trimipramine	1.18	2.51
	Pethidine	1.25	2.72
	Clomipramine	1.27	2.78
0.10% (v/v) (9.25 m*M*)***	Lorazepam	0.40	0.38
	Oxazepam	0.47	0.62
	Desethylamiodarone	0.49	0.69
	Temazepam	0.51	0.76
	Nordiazepam	0.52	0.80
	Maprotiline	0.55	0.90
	Prazepam	0.60	1.07
	Diazepam	0.61	1.11
	Amiodarone	0.65	1.25
	Nomifensine	0.65–0.84	1.25–1.90
	Butriptyline	0.67	1.32
	Promethazine	0.68–0.78	1.35–1.70
	Chlormethiazole	0.73	1.52
	Morphine	0.74–0.96	1.56–2.32
	Amitriptyline	0.75	1.59
	Atropine	0.75–0.86	1.59–1.97
	Dothiepin	0.76	1.63
	Fenethazine	0.76–0.89	1.63–2.08
	Phenazone	0.76–0.82	1.63–1.83
	Imipramine	0.77	1.66
	Amidopyrine	0.78–0.96	1.70–2.32

(Continued on p. 34)

TABLE I *(continued)*

Perchloric acid concentration	*Compound*	*Relative retention time*	*k'**
	Doxepin	0.78	1.70
	Mianserin	0.79	1.73
	Codeine	0.80–1.04	1.77–2.59
	Medazepam	0.80	1.77
	Orphenadrine	0.81	1.80
	Chlorpromazine	0.82	1.83
	Dihydrocodeine	0.85–1.13	1.94–2.91
	Flurazepam	0.87–1.15	2.01–2.97
	Dibenzepine	0.93	2.21
	5,6-Benzoquinoline (internal standard)	1.00 (= 5.53 min)	2.39
	Quinidine	1.00–1.55	2.39–4.36
	Disopyramide	1.02–1.60	2.53–4.53
	Procainamide	1.14–1.44	2.94–3.98
	Quinine	1.22–1.89	3.22–5.53
	Zimelidine	1.29–3.27	3.46–10.3

* Void volume of column measured from the injection point to the first deviation of the baseline following the injection of 100 μl of acetone.

** Some additional compounds giving rise to tailing peaks with this eluent are listed subsequently.

*** Compounds giving rise to tailing peaks indicated by range of values corresponding to the analysis of 100 μg and 1 μg, respectively.

tems may also be used at relatively low UV wavelengths, such as 215 nm, and this is illustrated by the analysis of the cardioactive drugs tocainide, mexiletine, monoethylglycinexylidide (desethyllignocaine), R17251 (internal standard) and lignocaine (Fig. 15). (The isothermal GLC analysis of these compounds is not possible owing to molecular weight differences). Electrochemical oxidation detection may also be employed (Fig. 2; see also ref. 9), although some analytes may not respond at strongly acidic eluent pH values. On the other hand, the use of non-aqueous eluents does permit the use of higher applied potentials than is possible using aqueous mobile phases.

Further considerations common to both qualitative and quantitative analysis are that most analytes give rise to sharp, symmetrical peaks with appropriate non-aqueous ionic eluents. Indeed, much higher efficiencies can be attained than is possible with basic compounds by "reversed-phase" separations with aqueous eluents, and this enhances not only resolution from potentially interfering compounds but also the accuracy of retention-time measurements and the limits of accurate measurement in quantitative analyses. The systems give excellent performance at ambient temperature, and the inadvertent addition of water has little effect (*cf.* Fig. 9).

Observations on the retention mechanism

It is clear from the studies of the influence of eluent pH on retention that only ionized bases are retained, and this together with the subsequent effect of ionic strength suggests that the retention mechanism must be based on ionic interactions. Both ion-pair formation[18] and ion exchange[19] have been suggested previously as the

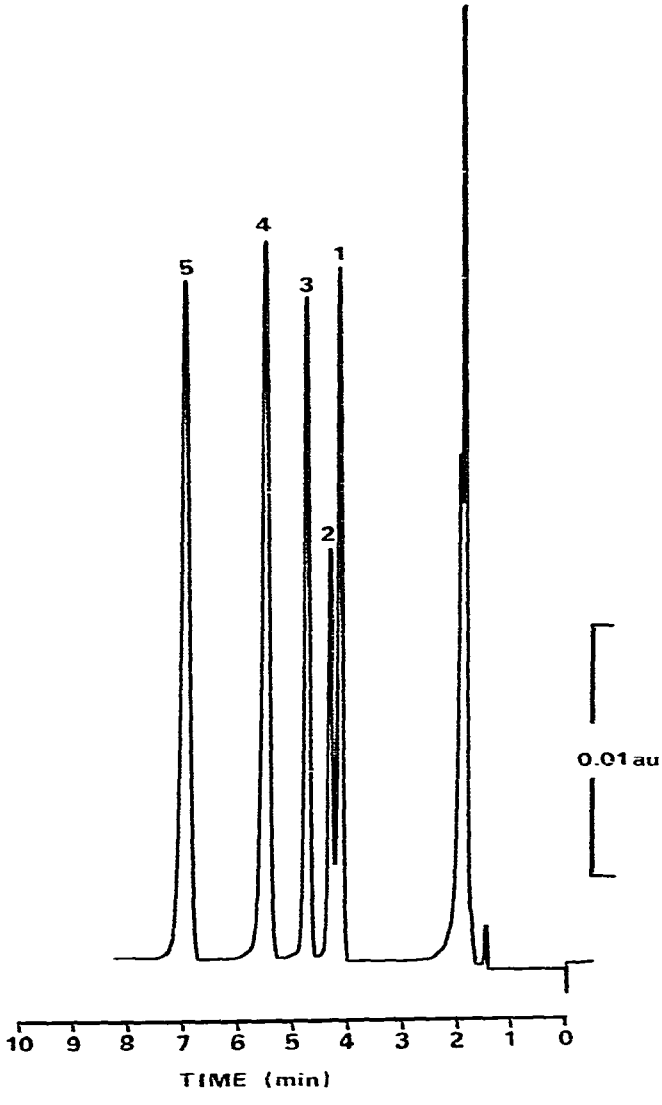


Fig. 15. Chromatography of tocainide (1), mexiletine (2), monoethylglycinexylidide (3), R17251 (internal standard) (4) and lignocaine (5) on a 250-mm column packed with Spherisorb 5 silica. Eluent: methanol containing perchloric acid (0.02%, v/v; 1.85 m*M*). Flow-rate: 2.0 ml/min. Detection: UV, 215 nm. Injection: 100 μl of methanolic solution containing tocainide and mexiletine (both 2 mg/l) and the remaining compounds (all 4 mg/l).

major retention mechanism of basic drugs on silica using highly methanolic eluents. It is likely therefore that retention is mediated in the first instance by ionized silanol groups on the silica surface since silica gel is weakly acidic in character. This would explain the non-elution of relatively strong bases such as amiodarone when using methanol alone, and also the lower ionic strength required to promote the elution of amiodarone at a given retention time under strongly acidic conditions (apparent pH

< 0) than under more moderate conditions (pH 4) (Fig. 5), since more silanol groups would be ionized and thus available to retain the analyte under these latter conditions.

Evidence against an ion-pair mechanism is provided by the observation that, if the ionic strength is held constant, the addition of water to the eluent has little effect on retention: an ion pair would be expected to be less soluble in aqueous solution than in methanol. Similarly, the fact that the nature of the competing anion has little overall effect on retention argues against an ion-pairing mechanism, although the absence of an effect attributable to the nature of the competing cation argues against ion exchange. The observed variation of retention of 1°, 2° and 3° amines with pH on silica columns (Fig. 4) is compatible with either mechanism. Finally, the observed order of elution (1° < 2° < 3°) of a series of amines at pH 4.0 (*i.e.* all fully ionized) could be due to lower solubility of the more hydrophobic ions in methanol or to differences in the solvation of the ion. The elution order for these compounds is thus compatible with an ion-exchange system since retention is normally greatest for ions, of comparable charge, that are least solvated, *i.e.* the more hydrophobic ions.

Thus, although the mechanism of retention is obviously based on ionic interactions, whether ion exchange or ion-pair formation predominates is not clear from these observations. Further studies may provide information to clarify the issue, and may also aid the interpretation of results obtained using silica columns with primarily aqueous eluents[13–16].

CONCLUSIONS

The addition of inorganic or organic ionic modifiers at relatively low concentrations to organic eluents can promote the elution of a variety of basic compounds from silica or bonded-phase silica columns.

Characteristics of such systems are (i) only ionized bases are retained, (ii) the retention time is generally dependent on the ionic strength and pH of the eluent and (iii) for a range of structural analogues those compounds of apparently greater polarity elute before the remaining compounds.

The degree of retention and the selectivity of the system can be influenced by changes in (i) the pH of the eluent, (ii) the ionic strength, (iii) the composition of the organic component of the eluent and (iv) the stationary phase.

These non-aqueous ionic systems show high efficiency, stability and reproducibility and give long column-life, and their applicability using low-wavelength UV, fluorescence and electrochemical oxidation detection has been demonstrated.

Especially when used in conjunction with simple solvent-extraction procedures, such systems have many advantages over conventional "reversed-phase" chromatography using primarily aqueous eluents, and should be used preferentially in the analysis of basic drugs and other basic compounds.

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