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ANALYSIS OF SOME COMMERCIAL PREPARATIONS FOR MIGRAINE TREATMENT USING ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ADDITION OF SALTS TO THE MOBILE PHASE

GARY K. C. LOW and P. R. HADDAD*
Department of Analytical Chemistry, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033 (Australia)
and
A. M. DUFFIELD
Biomedical Mass Spectrometry Unit, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033 (Australia)
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SUMMARY

When appropriate salts are added to the mobile phase in ion-pair high-performance liquid chromatography (HPLC) it is possible to arrive at an isocratic solvent with which complex mixtures of nitrogenous compounds of different pK_a values and lipophilic characteristics may be separated. Selectivity in the manipulation of solute retention depends on the salt type, its concentration, the percentage of organic modifier in the mobile solvent and the solute itself. In addition to a dramatic reduction in analysis time, the use of salts can also improve the resolution of closely eluted peaks. With judicious control of the pH of the mobile solvent, the addition of salts to the mobile phase can cause the retention of compounds of different pK_a values to alter in a contrasting manner. Under typical ion-pair HPLC conditions, an increase in salt concentration in the mobile solvent enhances the retention of neutral compounds and reduces the retention of ionized compounds. An inverse log–log relationship between the capacity factor of a solute and the salt concentration in the mobile phase was found. Examples are given of the use of salts in mobile solvents for ion-pair HPLC of a number of pharmaceutical preparations employed for the treatment of migraine.

INTRODUCTION

The analysis of compounds which contain amino groups is very important in biological and pharmaceutical applications. The conditions for separation of basic nitrogenous compounds by high-performance liquid chromatography (HPLC) have always been dependent on the chemistry of these bases[1]. Two important parameters regulating the elution profiles of these compounds from reversed-phase columns are their pK_a values and lipophilic characteristics. In the analysis of mixtures of bases which differ widely in these parameters, the most popular method for separation has

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been gradient elution ion-pair HPLC[2]. Whilst this is adequate for qualitative analysis, the lack of precision has limited its application. It has recently been demonstrated[3] that chromatographic separation with gradient elution can only be reproducible when isocapacitive mobile solvents are used. These solvents are defined as mobile phases in which the pairing ion concentration is similar to that in the stationary phase.

The addition of salts to mobile phases is a standard practice in ion-exchange chromatography of ionized solutes, where the ions from the salt compete for the charged sites on the ion-exchange resin. In salting-out chromatography, salts have been used for the elution of neutral organic compounds from ion-exchange columns. In an attempt to relate the retention of a solute to the concentration of the salt in the mobile solvent, the following empirical formula was proposed[4,5]

$$\log k' = \log k'_0 + K_s M$$

where k' and k'_0 are the capacity factors of a solute eluted using mobile solvents with and without salt, respectively, M is the concentration of the salt in the mobile solvent and K_s is the salting-out constant. This expression is of the same form as the well known Setchenow equation[6] which describes the effects of salts on the solubility of non-electrolytes in aqueous solution. The salt constants in both equations depend on the nature of the salts used.

The use of salts in mobile solvents has been successfully applied to the separation of small neutral or ionized compounds on reversed-phase columns[7–10]. The enhancement of retention of neutral compounds with increasing salt concentration has been explained by the solvophobic theory[7] in terms of the combined effects of a reduction of electrostatic repulsion between solute molecules, an increase in surface tension of the eluent and the concomitant increase in the energy required for cavity formation to accommodate the solute molecule in the solvent. For ionized solutes, a more complicated relationship is involved: at low salt concentrations, the retention decreases with increasing salt concentration, whereas at sufficiently high salt concentration, an increase of salt in the mobile solvent increases retention[8]. Jandera *et al.*[9] have demonstrated that the solvophobic theory cannot be strictly applied to these retention phenomena.

Until recently, the addition of salts in ion-pair chromatography had been employed only to assist in the elucidation of the retention mechanism of ion-pair chromatography. A number of authors[10–12] have suggested that an inverse dependence of the capacity factor on the salt concentration in the mobile solvent can be used to support the dynamic ion-exchange hypothesis, however the same relationship is essentially consistent with a variety of alternative retention mechanisms[13–15]. In these studies, the solutes used have generally been small molecules such as biogenic amines and amino acids, and no allowances have been made for selectivity differences arising from the use of different salts.

In this report we discuss a number of attractive features of the use of salts in ion-pair HPLC of nitrogenous basic drugs, in particular with regard to selectivity in retention reduction and improvement in the resolution of closely eluting peaks. With judicious control of the pH of an isocratic mobile solvent containing a salt, it is possible to analyse a number of pharmaceutical preparations employed for migraine.

These preparations generally contain nitrogenous bases of varying pK_a and lipophilic properties, and the addition of salts to mobile solvents not only reduces analysis time but can also exert opposite effects on the retention of neutral and ionized molecules. The relationship of the capacity factors of a number of these nitrogenous drugs to the salt concentration in the mobile phase of ion-pair HPLC is also investigated.

EXPERIMENTAL

Standards and reagents

The standard drugs were obtained from various sources: (±)-ephedrine hydrochloride, caffeine and theophylline from Sigma (U.S.A.); ergotamine tartrate from Fluka (Switzerland) and cyclizine hydrochloride, meclozine hydrochloride and diphenhydramine hydrochloride from the National Biological Standards Laboratory (Canberra, Australia). These materials were shown to be free from contaminants by gas chromatographic–mass spectrometric (GC–MS) analysis and test solutions were made up in methanol–water (50:50) at a concentration of 1.0 mg/ml.

Analytical grade ammonium sulphate, lithium sulphate, magnesium sulphate, sodium sulphate and sodium chloride were purchased from BDH (Australia). The salts were washed with anhydrous methanol and dried in a desiccator under vacuum before use. *n*-Heptanesulphonic acid (sodium salt) and acetic acid were obtained from Ajax Chemicals (Australia) and used without further purification.

Analysis of pharmaceutical formulations for migraine treatment

Four different formulations were purchased over the counter in Sydney, Australia. The ingredients and their concentrations in each preparation are shown in Table I. Twenty randomly selected tablets from each of the four formulations were crushed and an accurately weighed portion of powder equivalent to the average weight of a single tablet was dissolved in an appropriate volume of methanol–water

TABLE I

QUANTITATION OF SOME COMMERCIAL PHARMACEUTICAL PREPARATIONS FOR MIGRAINE TREATMENT

Preparation	*Manufacturer*	*Stated concentration of active ingredients (mg)*	*%* of stated content*
Cafergot	Sandoz (Switzerland)	Ergotamine tartrate, 1.0	99 ± 1.2
		Caffeine, 100	101 ± 0.5
Ergodryl	Park Davis (Australia)	Ergotamine tartrate, 1.0	99.0 ± 1.0
		Caffeine citrate, 100	99.6 ± 1.2
		Diphenhydramine hydrochloride, 25	95.0 ± 1.3
Ergalan	Allen and Hanburys (Australia)	Ergotamine tartrate, 1.0	94.5 ± 2.0
		Caffeine, 100	99.0 ± 0.8
		Meclozine hydrochloride, 10	100.0 ± 1.0
Migral	Burroughs Wellcome (Australia)	Ergotamine tartrate, 2.0	98.0 ± 2.0
		Caffeine hydrate, 100	101.0 ± 1.3
		Cyclizine hydrochloride, 50	100.0 ± 1.2

* Results are expressed as the percentage of the declared content of the active ingredient ± standard deviation. The standard deviation is estimated from the range of three individual determinations.

(50:50) in a volumetric flask, with the aid of a ultrasonic bath. The solution was then filtered and diluted, if necessary, to an appropriate concentration for injection onto the HPLC column.

Quantitations were made by using the chromatographic peak heights, and the concentrations of active ingredients in each tablet formulation were calculated with respect to their stated contents. All assays were performed in triplicate.

Instrumentation and chromatographic procedures

The liquid chromatograph system consisted of a Waters Model M6000A solvent pump, Model U6K injector, Model M450 variable wavelength detector (Waters Assoc., Milford, MA, U.S.A.) and an Omniscribe Model B5217-1 recorder. The detector was generally operated at 254 nm with a sensitivity setting of 0.1 a.u.f.s., however for separations of drugs in pharmaceutical preparations the wavelength was 245 nm. Because of the large differences in concentrations of active ingredients in these formulations, the setting of the detector at this wavelength tended to bias the sensitivity of detection towards the drugs of low concentration. All separations were carried out at 20°C and the flow-rate was set at 2.0 ml/min unless otherwise indicated.

Separation by HPLC was accomplished using a μBondapak C_{18} column (30 cm × 4.7 mm I.D., Waters Assoc.). The column was equilibrated with each mobile phase before use; a constant retention time was usually obtained after pumping 20–30 column volumes. When changing from one mobile phase to another, the column was washed with 10 ml of 0.01 N H_3PO_4 (pH 3.0) and 15 ml of methanol–water (50:50 v/v) before equilibrating with the new solvent. When mobile solvents containing sodium chloride were used, the HPLC system was washed with water immediately after use. A number of similar C_{18} columns were used in this study, however the column was not changed until a particular aspect of the study was completed.

Preparation of mobile phases

Analytical grade methanol was triply distilled from all-glass apparatus and water was distilled using a Millipore Milli-Q water purification system. The mobile phases were usually prepared immediately before use by dissolving 5.0 mmole of heptanesulphonic acid (sodium salt) and an appropriate concentration of salt in methanol–water mixtures containing 1.0% (v/v) glacial acetic acid. All mobile solvents were prepared in single large batches of sufficient quantity for experiments on a particular aspect of the study, so that intrastudy variations were eliminated. The exact ingredients of the mobile phase used are given in the figure captions.

Mobile phases were aspirated through 0.7-μm glass microfibre paper filters (GF/F, Whatman), degassed in an ultrasonic bath and allowed to equilibrate to ambient temperature before use.

RESULTS AND DISCUSSION

Selection of solutes

The compounds selected for this study are listed in Table II together with their pK_a values and connectivity indices, χ[16]. The connectivity index is a useful indication of the lipophilic character of a molecule and is directly related to the cavity surface area, polarizability, solubility and partition coefficient of that molecule[17]. The calcu-

TABLE II

CONNECTIVITY INDICES AND pK_a VALUES FOR COMPOUNDS STUDIED

Name	*Connectivity index, χ*	*pK_a**
Caffeine	5.37	< 1.0
Cyclizine	8.34	8.16
Diphenhydramine	8.27	9.12
Ephedrine	5.25	10.8
Ergotamine	12.12	6.4
Meclozine	9.41	–
Theophylline	5.13	< 1.0

* From ref. 25.

lated values of χ reported here are approximate, in that no corrections were made for the contributions of different functional groups, such as carbonyl and hydroxyl, in the molecule.

The compounds shown in Table II are widely distributed in their pK_a and connectivity index values. Under the mobile phase pH conditions used in this study (pH 3.5), theophylline and caffeine remain essentially neutral, whereas all of the other species are in their protonated forms. In view of the large differences in χ values, the separation of a mixture of these compounds, *e.g.*, caffeine, ephedrine and ergotamine, would be difficult under isocratic conditions. Ion suppression methods[18] cannot be used here since the pH required would be in excess of that permissible with silica columns.

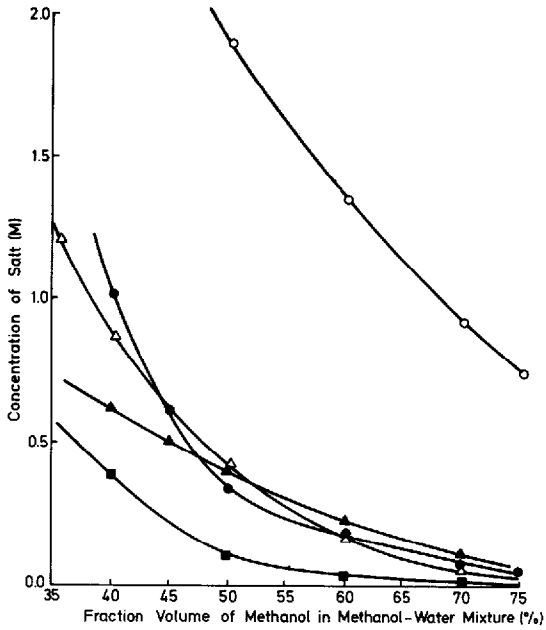

Fig. 1. Variation of the solubility of salts with changes in the fraction of methanol in methanol–water mobile solvents. ○ = NaCl; ● = $MgSO_4$, △ = $(NH_4)_2SO_4$; ▲ = Li_2SO_4; ■ = Na_2SO_4.

Salt effects

The solubilities of the various salts studied in methanol–water mixtures were determined experimentally by dissolving a known excess of salt in the solvent and accurately weighing the dried residue. Fig. 1 shows the solubilities of these salts at 20°C.

The effects of addition of the salts NaCl, $MgSO_4$, $(NH_4)_2SO_4$, Li_2SO_4 and Na_2SO_4 to the mobile phase in ion-pair HPLC on the retention behaviour of the solutes ergotamine, cyclizine, diphenhydramine, ephedrine and caffeine are shown in Fig. 2. For the first four compounds, inclusion of a salt in the mobile phase caused a reduction in retention, whereas for caffeine the retention was either unaffected or slightly increased. The different behaviour of caffeine is clearly due to the fact that it is not ionized under the pH conditions used. Similar results were obtained with theophylline, which is also not ionized. The general pattern observed for the protonated solutes was that a sharp decrease in retention occurred upon the initial addition of salt, after which only slight changes occurred with further addition of salt.

The results given in Fig. 2 indicate that the observed change in retention is strongly dependent on the type of salt added to the mobile phase. The general order of efficiency with which the salts reduced the retention of the solutes tested was $Li_2SO_4 > (NH_4)_2SO_4 > NaCl > Na_2SO_4 > MgSO_4$, although the order did vary slightly depending on the nature of the solute. At higher concentrations of salt than those shown in Fig. 2, a slight increase in retention was observed (Fig. 3) and this has been rationalized[13] in terms of the gradual dominance of the surface tension terms in the retention equation developed from the solvophobic theory. The same phenomenon has also been attributed to salting-out effects caused by the increased concentration of salt in the mobile phase[18].

The order of efficiency of retention reduction exhibited by the various salts is different for ephedrine than for the other protonated species. This difference must be

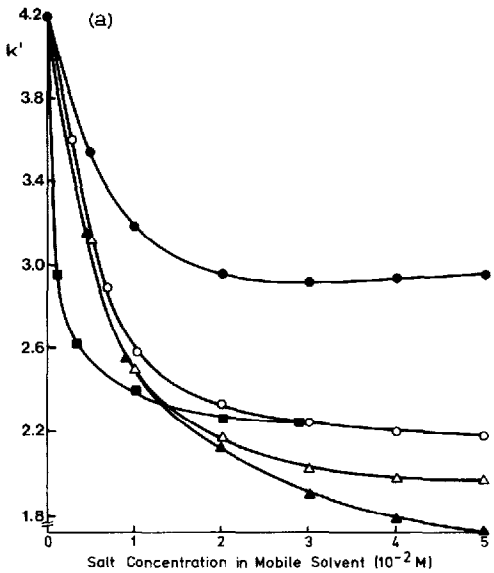

Fig. 2.

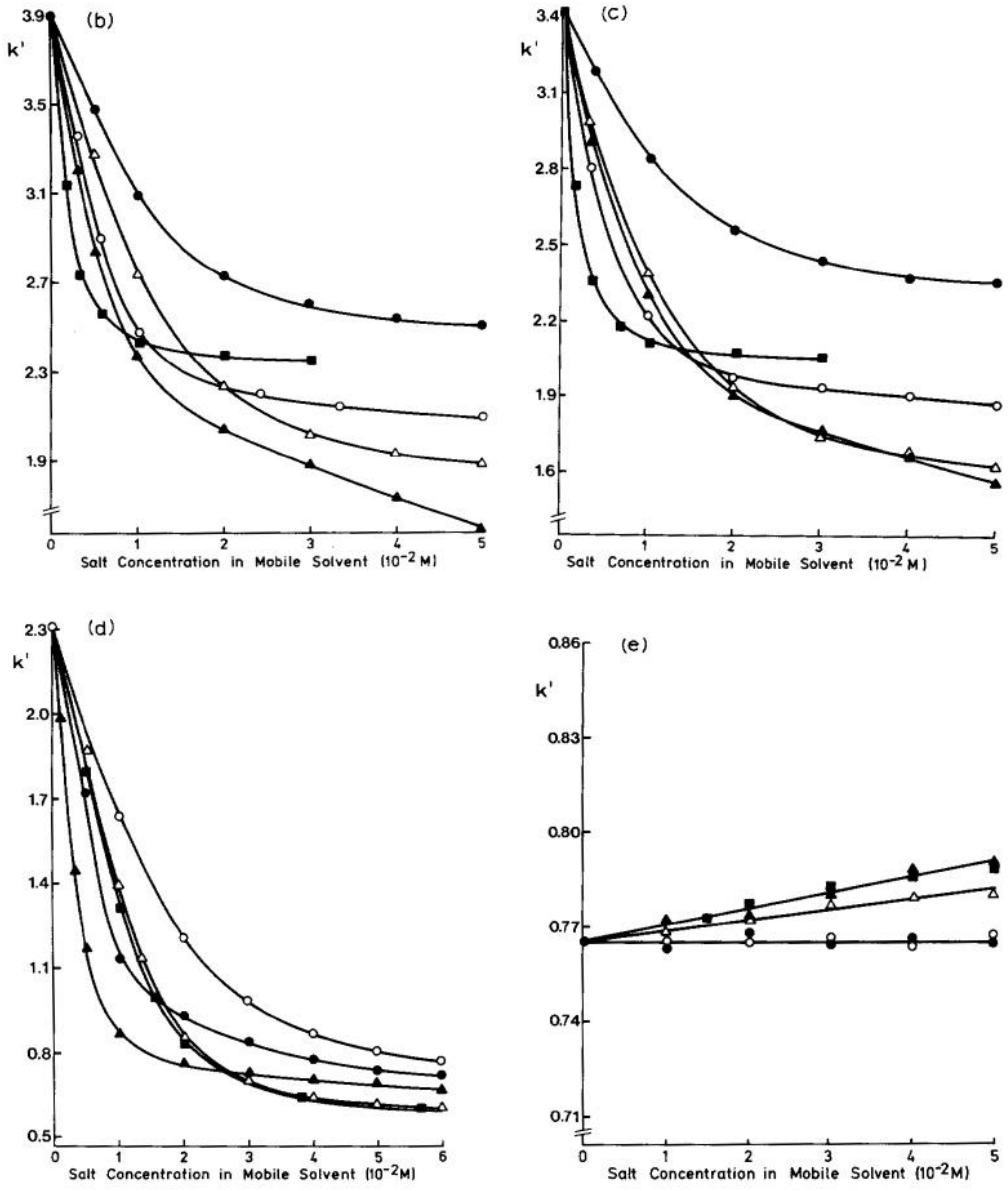

Fig. 2. Change of the capacity factor, k', with salt concentration in the mobile solvents. Salts are denoted as in Fig. 1. Solutes: a, ergotamine; b, cyclizine; c, diphenhydramine; d, ephedrine; e, caffeine. Mobile solvents: a–c, methanol–water (60:40) containing 5.0 mM sodium heptanesulphonate and 1.0% acetic acid; d, e, methanol–water (43:57) containing 5.0 mM sodium heptanesulphonate and 1% acetic acid.

partially attributed to the less lipophilic nature of ephedrine compared with the other compounds. A lower fraction of organic solvent was employed in the mobile phase used for ephedrine in order to obtain a workable k' value which would illustrate salt effects ($k' > 1.5$ at zero salt concentration).

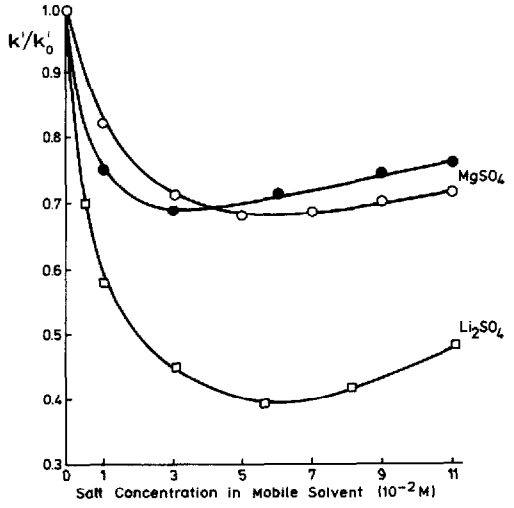

Fig. 3. Examples of the increase in retention at higher salt concentration in the mobile solvent. Mobile solvent as for Fig. 2a–c. □, ○ = Diphenhydramine; ● = ergotamine. k'_0 is the capacity factor obtained without addition of salt to the mobile phase.

The effect of added salt was somewhat dependent on the proportion of methanol present in the mobile phase. In mobile phases containing low amounts of methanol the k' value reached a lower plateau level than that observed for higher amounts of methanol. In addition, the initial rapid decrease in k' occurring upon the first addition of salt was more dramatic in mobile phases containing higher proportions of methanol. This trend is expected since the concentration of pairing ion adsorbed onto the stationary phases decreases as the fraction of organic modifier is increased. Competition by added salt ions can therefore more readily reduce the retention of an ionized solute.

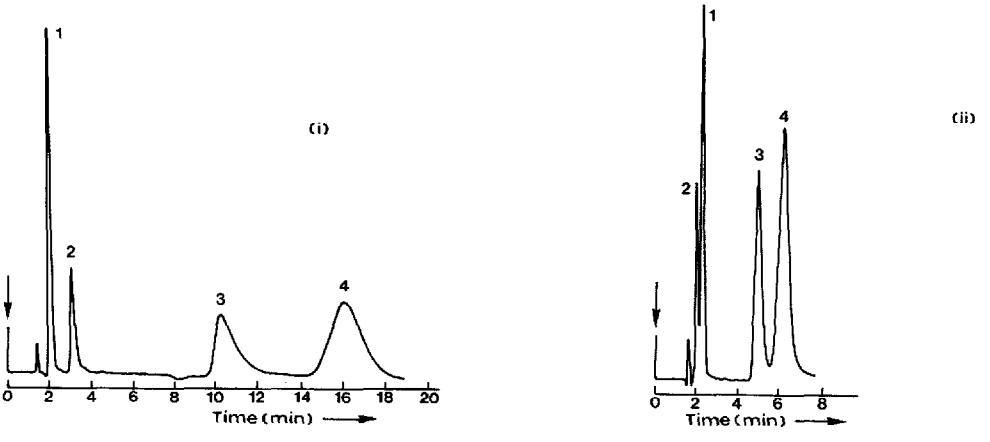

Fig. 4. Comparison of isocratic separations of synthetic mixtures of caffeine (1), ephedrine (2), diphenhydramine (3) and ergotamine (4) with two different mobile solvents. Mobile solvents: i, methanol–water (50:50) containing 5.0 mM sodium heptanesulphonate and 1.0% acetic acid; ii, as for i except with $7.0 \cdot 10^{-2}$ M Na_2SO_4 added.

Selectivity and resolution

The possibility of using the addition of salt to selectively manipulate retention is evident from the data in Fig. 2. It is clear that the retention of a solute in ion-pair HPLC is a function of the combined effects of the added salt type, salt concentration, the fraction of organic modifier in the mobile phase and the nature of the solute. An example of selective retention manipulation is given in Fig. 4 which depicts the isocratic separation of a mixture of caffeine, ephedrine, diphenhydramine and egotamine using mobile phases both with and without the presence of added salt. Addition of Na_2SO_4 resulted in a decreased analysis time, a reversal of the elution order of caffeine and ephedrine and an improvement in peak shape for all solutes.

The resolution of closely eluted peaks is also governed by the concentration of salt in the mobile phase. We have found that an increase in salt concentration leads to an increase in resolution, followed by a decrease at higher salt concentrations. This effect was particularly pronounced with columns of low efficiency and can be explained in terms of the binding of exposed silanol sites by the salt in the mobile phase. The results are illustrated in Fig. 5 which shows that the resolution of ergotamine and

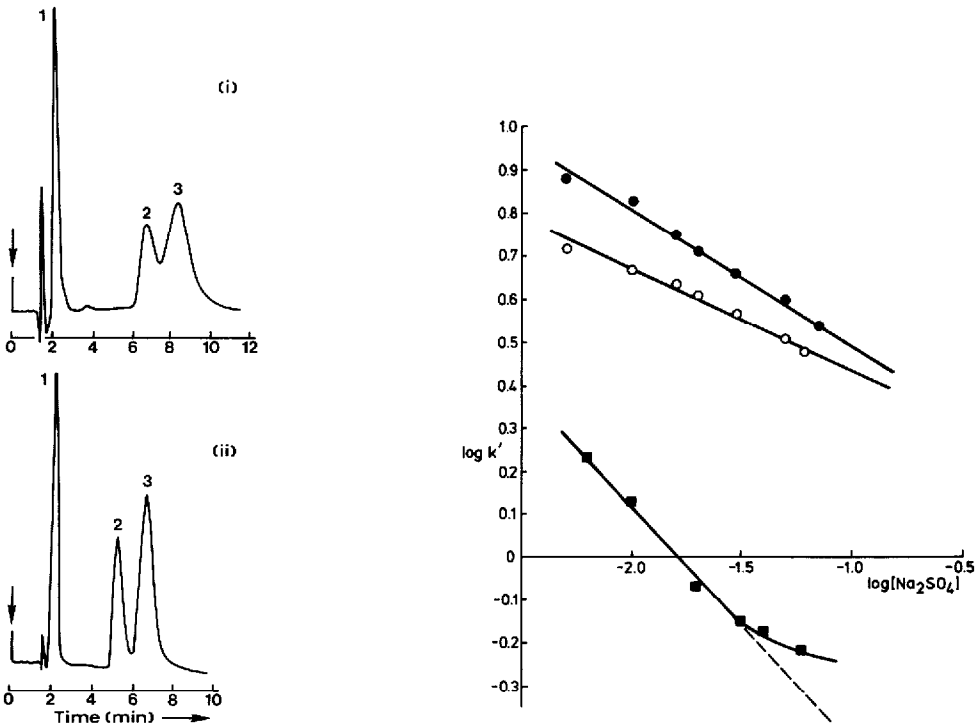

Fig. 5. An example of the improvement in the resolution of two closely eluted peaks produced by changing the salt concentration in the mobile solvent. Salt concentrations: i, $0.71 \cdot 10^{-2}$ *M*; ii, $2.63 \cdot 10^{-2}$ *M* in methanol–water (50:50) containing 5.0 m*M* sodium heptanesulphonate and 1.0% acetic acid. Peaks: 1 = caffeine; 2 = diphenhydramine; 3 = ergotamine.

Fig. 6. Observed log–log relationship between k' and Na_2SO_4 concentration in the mobile solvent. Mobile phase: methanol–water (50:50) containing 5.0 m*M* heptanesulphonate, 1% acetic acid and the indicated concentration of Na_2SO_4. ● = Ergotamine; ○ = diphenhydramine; ■ = ephedrine.

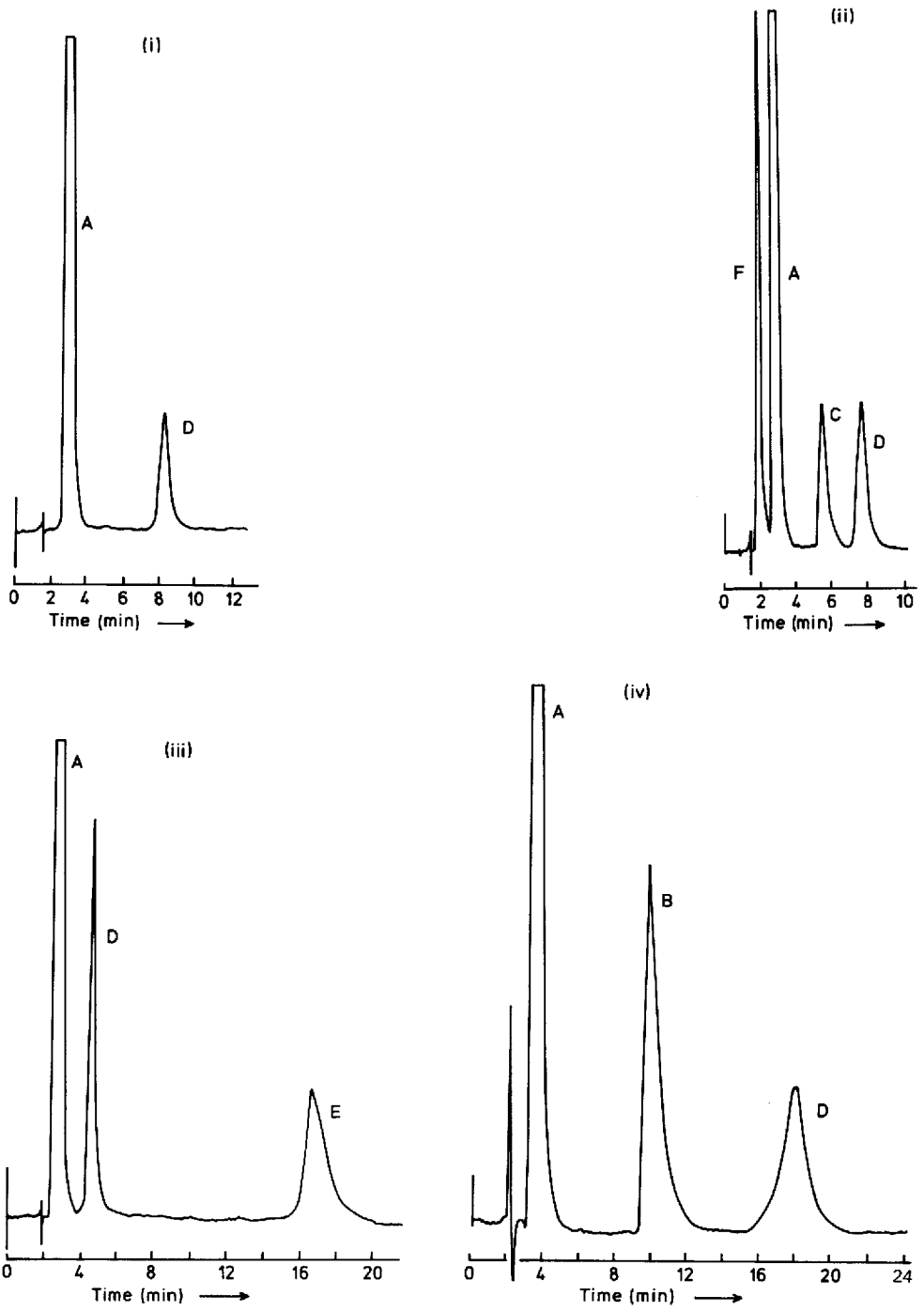

Fig. 7. Separations of pharmaceutical preparations for migraine treatment using methanol–water mobile solvents containing 5.0 m*M* sodium heptanesulphonate and 1.0% (v/v) acetic acid. i, Cafergot tablets, methanol–water (50:50) and $7.0 \cdot 10^{-2}$ *M* Na_2SO_4; ii, Ergodryl tablets, methanol–water (50:50) and $9.0 \cdot 10^{-2}$ *M* Na_2SO_4; iii, Ergalan tablets, methanol–water (51:43) and $4.0 \cdot 10^{-2}$ *M* Na_2SO_4; iv, Migral tablets, methanol–water (43:57) and $10 \cdot 10^{-2}$ *M* Na_2SO_4. Peaks: A = caffeine; B = cyclizine; C = diphenhydramine; D = ergotamine; E = meclozine; F = citric acid.

diphenhydramine is dramatically improved by an increase in the salt concentration, despite the fact that the retention of each species has been reduced. Addition of salt improves the resolution by narrowing the peaks, since the separation factors calculated from each of the chromatograms in Fig. 5 are almost identical (1.31 and 1.37 for Fig. 5i and ii, respectively). Improvements in resolution resulting from addition of salt to the mobile phase in ion-pair HPLC have been reported previously[20].

Relationship between k′ and [salt]

A number of explanations have been advanced for the mechanism by which addition of a salt to the mobile phase produces a reduction in retention in ion-pair HPLC. The uncertainty surrounding this mechanism is a direct result of the fact that considerable ambiguity still exists regarding the mechanism of ion-pair chromatography itself[14,15,20–24]. In many of the published studies, an inverse relationship has been reported between k' and the concentration of salt in the mobile phase[11,12,14,15]. We have experimentally determined the relationship between k' and [salt] for the solutes ephedrine, ergotamine and diphenhydramine; the pairing-ion concentration was maintained at 5 m*M*. The results of this study showed that, for the particular solutes and salt concentrations examined, the capacity factor was not inversely proportional to [salt]; rather an inverse log–log relationship was observed, as indicated by Fig. 6. The reasons for the disparity between these results and those previously reported could only be determined by further study, however we have used much larger solute molecules and a wider range of salt concentrations than in previous studies. These factors are likely to have a considerable effect.

Analysis of pharmaceutical preparations

To illustrate the utility of isocratic ion-pair HPLC with addition of salt to the mobile phase, several preparations employed for migraine treatment containing different mixtures of the test solutes were analysed. The salt concentration and the percentage of organic modifier in the mobile phase were adjusted to optimize the separation in the minimum analysis time. As far as possible, the salt concentration was maintained in the plateau region (see Fig. 2) to minimize variations in retention caused by slight changes in salt concentration. The chromatograms obtained are given in Fig. 7 and the results are summarized in Table I. These results clearly show that the proposed method of analysis has direct application to the analysis of such pharmaceutical formulations.

CONCLUSIONS

This study has demonstrated that the addition of salts to the mobile phase used in ion-pair HPLC can lead to selective changes in retention of solutes. The type of salt, the degree of ionization of the solute, the percentage of organic modifier, the salt concentration and the nature of the solute itself all play a part in determining solute retention. Adjustment of the salt concentration in the mobile phase can permit isocratic separation of a series of compounds previously separable only with gradient elution ion-pair HPLC.

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