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Optimization of the mobile phase for the liquid chromatographic separation of modafinil optical isomers on a Chiral-AGP column

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ABSTRACT

Optical isomers of modafinil were separated by chiral liquid chromatography on a Chiral-AGP column. Optimization of the mobile phase was undertaken in order to obtain a complete resolution of the two isomers. Two buffers, sodium dihydrogenphosphate and ammonium acetate, were studied and the influence of ionic strength and pH was investigated. The results showed that ionic strength was not a major parameter for optimization but that pH was. Ammonium acetate was a considerably better buffer than sodium dihydrogenphosphate. Different organic modifiers, ethanol, 1-propanol, 2-propanol, 1-butanol and 1-pentanol were studied with the two buffers. The best separations were obtained with 1-butanol and 1-pentanol.

INTRODUCTION

Modafinil, 2-[(diphenylmethyl)sulphinyl]acetamide, is an awakening drug [1], recently developed as the racemate; its structure contains an asymmetric sulphur atom. In order to study separately the different properties of pure optical isomers, they were synthesized and an analytical method was needed to assess their optical purity. This method might also be usable for pharmacokinetic studies. The best techniques to achieve these objectives seemed to be chromatographic methods, but gas chromatography failed because of the thermal lability of modafinil. Only liquid chromatography produced satisfactory results.

Different assays using a column packed with *d*-phenylglycine bound to silica gel were attempted using normal-phase high-performance liquid chromatography (HPLC) but failed; the best value of the separation factor was 1.03 for k' values around 20 [2]. Better results were obtained using a column packed with human α-1-acid glycoprotein bounded to silica gel and eluted with a mixture of potassium dihydrogenphosphate buffer and 1-propanol [2]. These chromatographic conditions, coupled with UV detection, were suitable for optical purity assessment, the purpose for which they were developed, but failed for pharmacokinetic purposes because of the lack of sensitivity and selectivity.

In order to circumvent these limitations, the different parameters which could improve a chromatographic separation were investigated bearing in mind that, for modafinil, the sensitivity and selectivity could be increased by using mass spectrometry (MS) with a thermospray (TSP) interface as a detector [3]. For this reason, potassium dihydrogenphosphate buffer was replaced with ammonium acetate buffer and different organic modifiers were tried.

This paper reports the optimization of the two mobile phases (dihydrogenphosphate and acetate) and their comparison.

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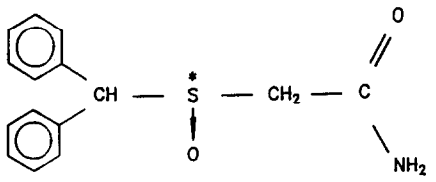

Fig. 1. Formula of modafinil.

EXPERIMENTAL

Standards

Modafinil, the structure of which is given in Fig. 1, and its two optical isomers CRL 40982 (−) and CRL 40983 (+), were synthesized in the research laboratories of Laboratoire L. Lafon (Maisons-Alfort, France). Standards solutions were prepared by dissolving separately 20 mg of modafinil and 10 mg of each isomer in 20 ml of methanol followed by 1:40 dilution 0.01 *M* KH_2PO_4. The volume of each solution injected was 20 μl, representing injected amounts corresponding to 0.9 nmol of each isomer.

Reagents and solvents

The reagents used to prepare buffer solutions, ammonium acetate, potassium dihydrogenphosphate and dipotassium hydrogenphosphate, were of analytical-reagent grade and were purchased from Prolabo (Paris, France). Acetic acid was purchased from UCB (Leuven, Belgium). Alcohols, used as organic modifiers, were of analytical-reagent or HPLC grade and were purchased from Prolabo and Merck (Darmstadt, Germany).

Equipment

The liquid chromatograph consisted of an SP 8780 autosampler equipped with a 20-μl loop, an SP 8800 ternary HPLC pump, an SP 8490 UV–visible programmable spectrophotometer operating at 220 nm and 0.2 a.u.f.s. and a Data Jet integrator, connected together through a Labnet Network (Spectra-Physics, Les Ulis, France). Chromatograms and data could be stored and treated on a Winner station (Spectra-Physics).

A Chromtech Chiral α AGP chromatographic column (10 cm × 4.6 mm I.D.) (Interchim, Montluçon, France) was thermostated with a Crococil insulated thermoregulated oven (Cluzeau, Ste. Foy la Grande, France). The flow-rate through the column was maintained at 1.0 ml/min and the temperature in the oven at 40°C throughout the study.

Experimental variables

Two different buffers were investigated: Potassium dihydrogenphosphate, which is recommended by the manufacturer of the Chiral α AGP column (but which is unusable in LC–TSP-MS, and ammonium acetate, which is usable with a TSP interface.

For each buffer, the following parameters were investigated, in this order: pH of the mobile phase; salt concentration; and nature and concentration of the organic modifier. The organic modifiers investigated were: ethanol, 1-propanol, 2-propanol, 1-butanol and 1-pentanol. Acetonitrile and methanol were found to be inefficient in separating optical isomers of modafinil in a previous study [2].

Measured parameters

For each set of chromatographic conditions, the column was equilibrated at a flow-rate of 1.0 ml/min until stable retention times were obtained for modafinil standard solution. The discrimination factor, d_0[a] [4] was measured from the chromatogram obtained, then CRL 40982 and CRL 40983 standard solutions were successively injected and the following parameters were measured simply with a millimetre graduated ruler: d_{R0}, "dead volume" distance of the chromatographic system, d_R, retention distance of the compound, and $b_{0,5}$, width at half-height.

From these values, the following parameters were calculated: k'_1, capacity factor of CRL 40982; k'_2, capacity factor of CRL 40983; α, separation factor, $\alpha = k'_2/k'_1$; R_s, resolution between CRL 40982 and CRL 40983; N_1, number of theoretical plates per metre for CRL 40982; and N_2, number of theoretical plates per metre for CRL 40983.

The values of N (and to a lesser extent R_s) must be treated with caution, as they were calculated from

[a] The discrimination factor, d_0, between two consecutive peaks was defined by El Fallah and Martin [4] as $d_0 = (h_p - h_v)/h_p$, where h_p is the distance from the top of the smallest peak to the baseline and h_v is the distance from the bottom of the valley between the two peaks to the baseline.

$b_{0.5}$ values for which the relative accuracy varied between 2 and 5%; consequently the relative accuracy of N and R_s ranged between 4 and 10% at least. For this reason, only variations of N and R_s larger than 15% should be considered.

RESULTS

During previous studies with potassium dihydrogenphosphate, we noticed that the efficiency of Chiral-AGP columns could drastically decrease because of the occurrence of a hole at the top of the column. Pouring in a small amount of Chiral-AGP slurry, to complete the bed of stationary phase, restored the column efficiency. Therefore, at the end of each study of the influence of one parameter, the column was re-equilibrated with the mobile phase used at the beginning of that study and standard solution was injected; if the values of d_R and $b_{0.5}$ had changed, the column was carefully checked, repacked if necessary and the experiments were repeated. If no hole was observed, the column was changed. In the phosphate buffer study, such a change was needed after the study of ionic strength. With acetate buffer, a change would occur after having studied the influence of basic pH, but this was done only after the ionic strength study.

Phosphate buffer

Influence of pH. Variations of mobile phase pH were obtained by mixing in different ratios a 0.01 *M* aqueous solution of KH_2PO_4 and a 0.01 *M* aqueous solution of K_2HPO_4, each solution containing 0.5% of 1-butanol as organic modifier. The ratios and corresponding pH values are listed in Table I.

Variations in k', α, R_s, d_0 and N *versus* pH are displayed in Fig. 2A–E, respectively. Small variations in the acidic or basic range did not influence the k' values but there was a net break around pH 7; the k' values for both compounds decreased by about 30%. Larger differences in k' values between CRL 40982 and CRL 40983 were obtained between pH 6 and 7. The break, described for k', was not observed for α values. To a first approximation, within the experimental accuracy, α increased linearly with pH from 1.05 to 1.17; the slope of the linear regression, calculated from experimental data, was equal to $3.31 \cdot 10^{-2}$ k' unit per pH unit.

TABLE I

INFLUENCE OF pH USING 0.01 *M* PHOSPHATE BUFFER: COMPOSITION OF THE MOBILE PHASE

Each KH_2PO_4–K_2HPO_4 mixture contained 0.5% of 1-butanol.

KH_2PO_4:K_2HPO_4	pH	KH_2PO_4:K_2HPO_4	pH
100:0	4.90	50:50	7.06
90:10	6.09	40:60	7.09
80:20	6.43	20:80	7.49
70:30	6.68	15:85	7.62
60:40	6.76	10:90	7.79

In the same way, R_s and d_0 increased linearly with pH in the acidic range but remained fairly constant in basic media. At pH 4.9, d_0 was equal to 0 as there was insufficient separation between CRL 40982 and CRL 40983 [4]. The highest values of R_s (0.79) and of d_0 (0.5) were obtained at pH 7.06. The number of theoretical plates varied in the opposite direction to k'. Variations of N *versus* pH showed complicated curves in which minimum values (about 5000 plates/m) occurred in acidic medium, and maximum values (about 12 000 plates/m) were observed at pH 7.5.

Influence of phosphate concentration. Variations of phosphate concentration were obtained by dilution of a 0.03 *M* aqueous solution of PO_4^{2-} at pH 7.06 with water. The PO_4^{2-} solution was a 50:50 mixture of a 0.03 *M* solution of KH_2PO_4 and a 0.03 *M* solution of K_2HPO_4. The final concentrations of PO_4^{2-} were 0.03, 0.02, 0.01 and 0.005 *M*. Each solution contained 0.5% of 1-butanol as organic modifier.

Variations in k', α, R_s, d_0 and N versus phosphate concentration are displayed in Fig. 3A–E, respectively.

The k' values decreased slowly, from 3.45 to 3.18 for CRL 40982 and from 3.96 to 3.68 for CRL 40983, when the phosphate concentration increased from 0.005 to 0.03 *M*. α (mean value 1.16), R_s (mean value 0.63) and d_0 (mean value 0.4) were independent of phosphate concentration. Variations in the number of theoretical plates were of the same order of magnitude as the experimental accuracy.

Influence of organic modifiers. The mobile phase used to study the influence of organic modifiers was 0.02 *M* phosphate solution (pH 7.06), prepared as

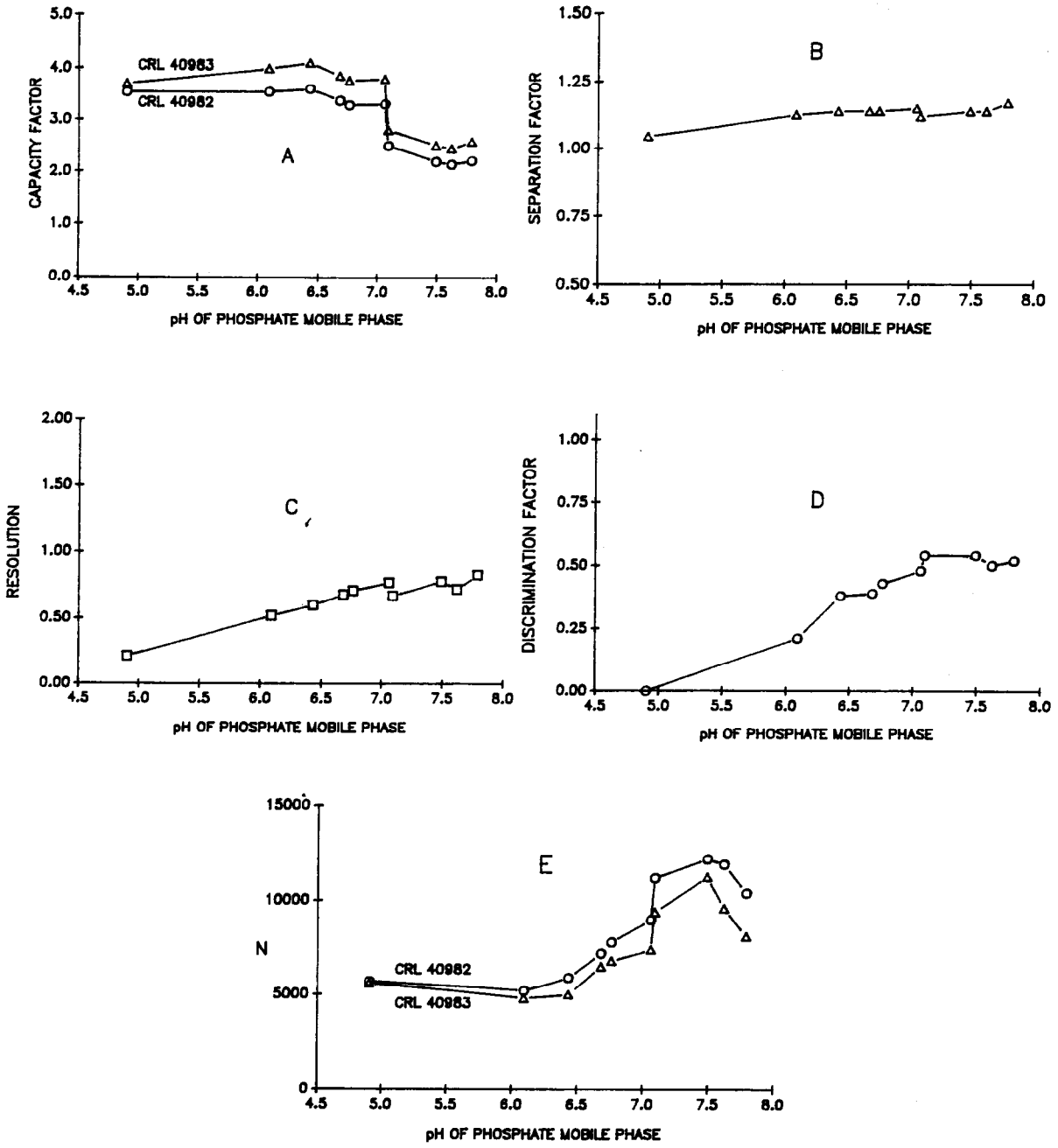

Fig. 2. Phosphate buffer: influence of pH on chromatographic parameters: (A) k'; (B) α; (C) R_s; (D) d_0; (E) N. Mobile phase, 0.5% 1-butanol in 0.01 M phosphate buffer (for composition, see text); flow-rate, 1.0 ml/min; column temperature, 40°C.

described above. In order to have sufficient accuracy, the amounts of organic modifiers were weighed and converted to volume using a density value of 0.8.

Variations in k' values with organic modifier concentration are displayed in Fig. 4A. The elution order was the same, CRL 40982 being eluted before

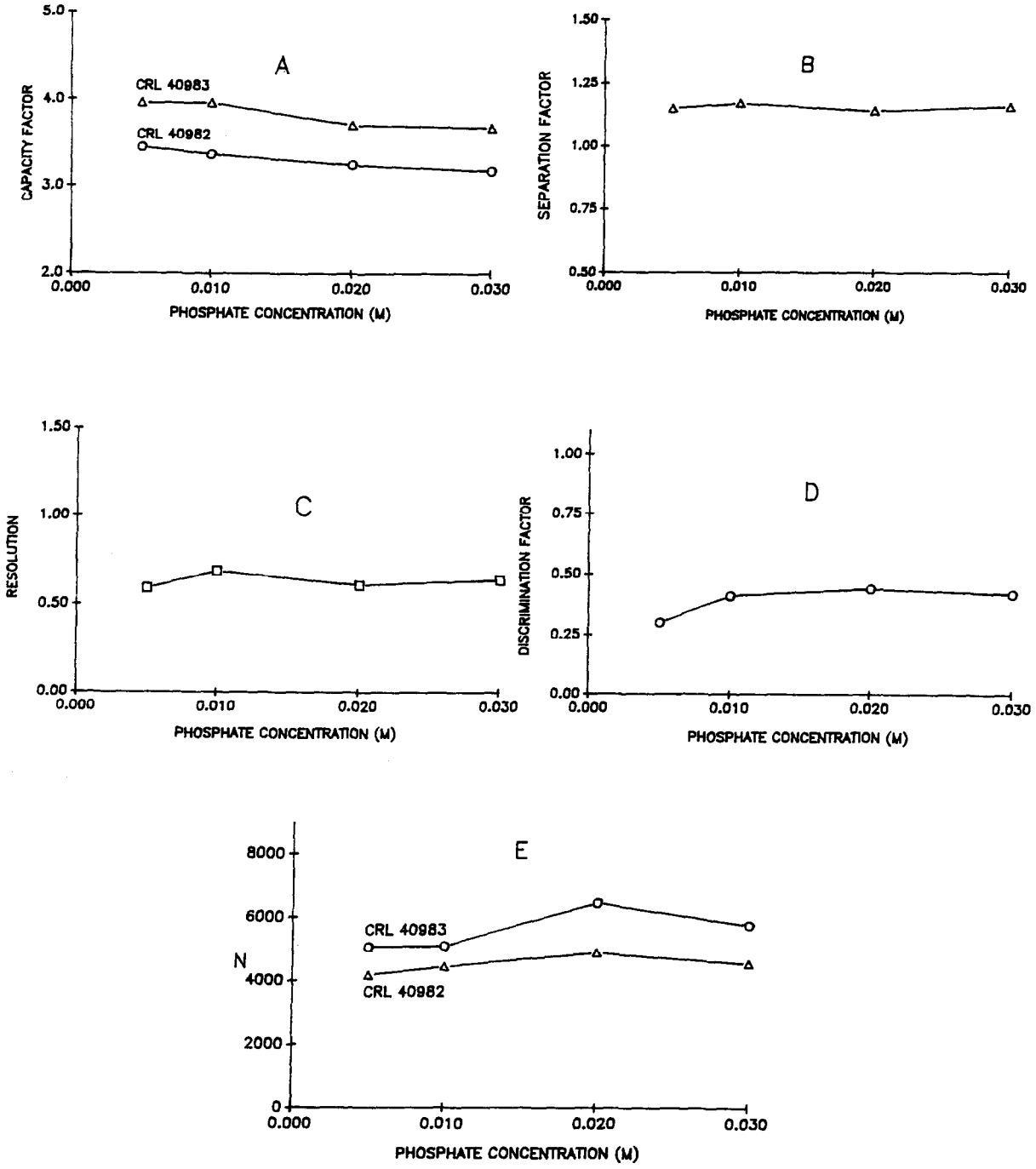

Fig. 3. Phosphate buffer: influence of phosphate concentration on chromatographic parameters. (A)–(E) as in Fig. 2. Mobile phase, 0.5% 1-butanol in phosphate buffer (pH 7) (for composition, see text); flow-rate, 1.0 ml/min; column temperature, 40°C.

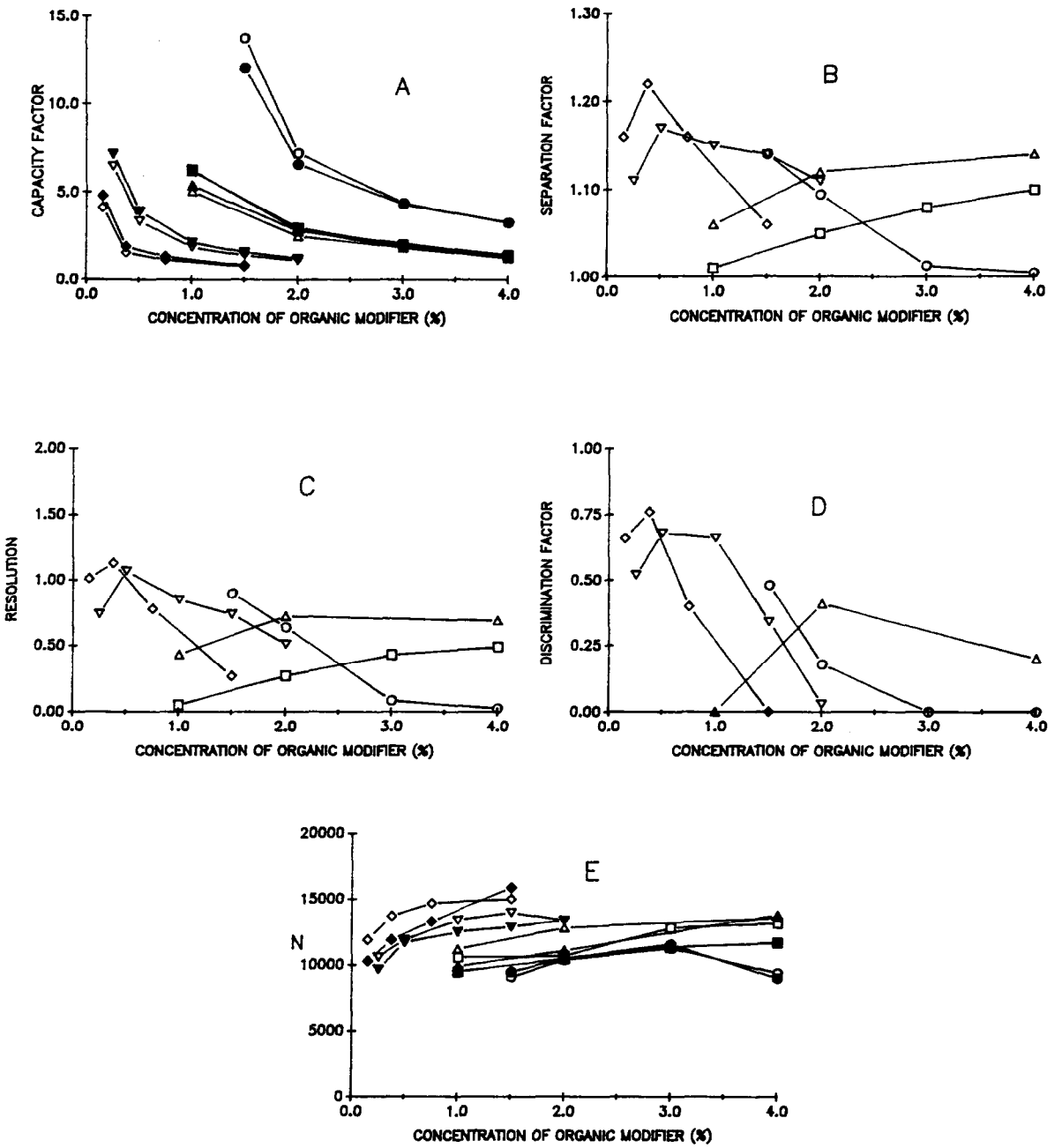

Fig. 4. Phosphate buffer: influence of nature and concentration of organic modifier on chromatographic parameters. (A)–(E) as in Fig. 2. Buffer of mobile phase: 0.02 *M* phosphate (pH 7); flow-rate, 1.0 ml/min; column temperature, 40°C. Open symbols, CRL 40982; closed symbols, CRL 40983. ○, ● = Ethanol; △, ▲ = 1-propanol; □, ■ = 2-propanol; ▽, ▼ = 1-butanol; ◇, ◆ = 1-pentanol.

CRL 40983 whatever the organic solvent, except with ethanol, for which the elution order was reversed. In each instance, the difference between the k' values of CRL 40982 and CRL 40983 was small, so the α values (Fig. 4B) ranged from 1.005 (4% ethanol) to 1.22 (0.375% 1-pentanol). The shape of the α curves varied with the nature of the modifier: for ethanol, the α values decreased from 1.14 to 1.005 when the ethanol concentration increased from 1 to 4%. For 1- and 2-propanol, the α values increased from 1.06 to 1.14 and from 1.01 to 1.10, respectively, when the modifier concentration increased from 1 to 4%. With 1-butanol as organic modifier, the α values reached a maximum (1.17) at 0.5% 1-butanol and then decreased slowly at higher concentrations. The same pattern was observed with 1-pentanol (maximum 1.22 at 0.375% 1-pentanol), but the decrease was faster.

The variations in R_s (Fig. 4C) followed the same kind of profile as described for α, except with 1-propanol, for which a maximum was reached at 2%. The observed R_s values were higher than 1.0 in only three cases: 1.07 for 0.5% 1-butanol and 1.01 and 1.13 for 0.15 and 0.375% 1-pentanol, respectively. The d_0 values (Fig. 4D), when they were different from zero, followed the same kind of pattern as R_s. In any case for 2-propanol, d_0 was equal to zero as no valley occurred between CRL 40982 and CRL 40983.

To a first approximation and in the studied range, the numbers of theoretical plates increased with increasing organic modifier concentration (Fig. 4E) but for ethanol, 1-propanol and 2-propanol their variations remained below or equal to the experimental accuracy. The variations in N values were greatest for 1-butanol and 1-pentanol. The highest values of N of 15 000 and 15 800 plates/m, respectively, for CRL 40982 and CRL 40983 were obtained with 1.5% 1-pentanol.

Acetate buffer

Influence of pH. Variations of mobile phase pH were obtained by mixing in different ratios a 0.1 M aqueous solution of ammonium acetate ($AcONH_4$) with a 0.1 M aqueous solution of acetic acid (AcOH), each solution containing 0.75% of 1-butanol as organic modifier. The pH of a 0.1 M aqueous solution of ammonium acetate being 6.75, higher values of pH were obtained by adding a few drops of concentrated ammonia solution to the 0.1 M ammonium acetate solution. The composition of the mobile phase and the corresponding pH values are listed in Table II.

Experimentally, pH was varied from 6.75 to 5.55 then, after re-equilibration, from 6.75 to 7.6.

The variations in k', α, R_s, d_0 and N with pH are displayed in Fig. 5A–E, respectively. The break in k' and N values observed in phosphate buffer at pH $\approx$ 7 did not occur in acetate buffer; in the studied range of pH, the k' of CRL 40982 was independent of pH (mean value 2.98) and the k' of CRL 40983 increased linearly with pH. Consequently, the α values increased linearly with pH; the slope of the linear regression, calculated from experimental results, was $5.82 \cdot 10^{-2}$ k' unit per pH unit.

To a first approximation, d_0 increased linearly with increasing pH, from 0.87 to 0.96. The R_s values also increased with pH, from 1.4 to 2.3. In contrast to the other parameters, the variations in the number of theoretical plates were almost independent of pH until pH 7.1 (mean value 22 000 plates/m for both CRL 40982 and CRL 40983).

At pH 7.6, the efficiency of the column began to decrease, indicating partial destruction of the col-

TABLE II

INFLUENCE OF pH USING 0.1 M ACETATE BUFFER: COMPOSITION OF THE MOBILE PHASE

Each mixture contained 0.75% of 1-butanol.

Component	Proportion (%)						
0.1 M $AcONH_4$	100	100	100	97.5	95	92.5	90
0.1 M AcOH	0	0	0	2.5	5	7.5	10
Conc. NH_3	$1 \cdot 10^{-4}$	$0.5 \cdot 10^{-4}$	0	0	0	0	0
pH	7.6	7.1	6.75	6.1	5.8	5.65	5.55

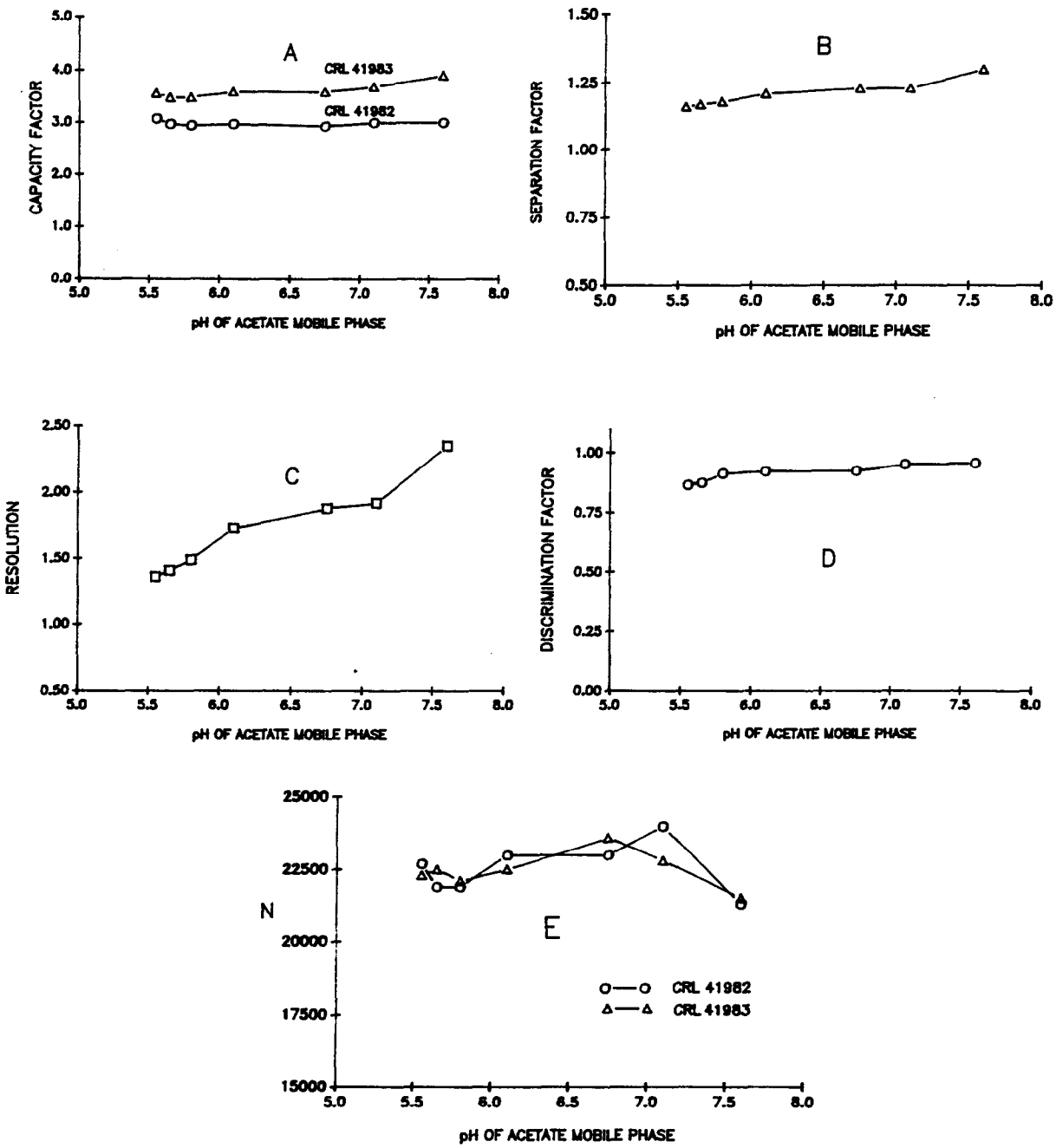

Fig. 5. Acetate buffer: influence of pH on chromatographic parameters. (A)–(E) as in Fig. 2. Mobile phase, 0.75% 1-butanol in 0.1 *M* acetate buffer (for composition, see text); flow-rate, 1.0 ml/min; column temperature, 40°C.

umn's qualities, observed at pH 7.8 (results at this pH were not reported because they were insignificant). The decrease in efficiency was irreversible even after a long re-equilibration with a mobile phase of acidic pH. Such a partial destruction was not observed with phosphate buffers, for which the

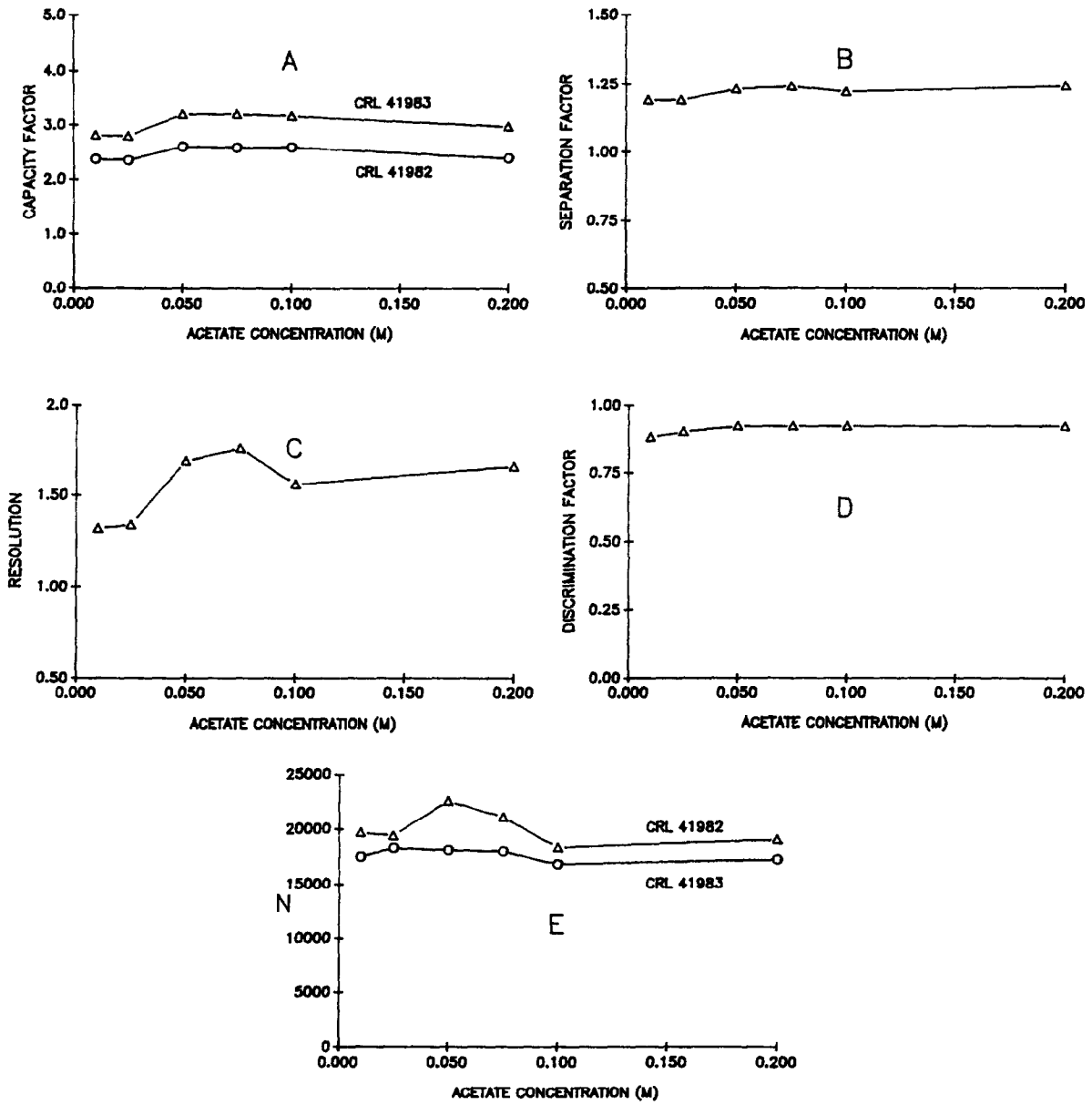

Fig. 6. Acetate buffer: influence of acetate concentration on chromatographic parameters. (A)–(E) as in Fig. 2. Mobile phase, 0.75% 1-butanol in ammonium acetate (pH 6.75); flow-rate, 1.0 ml/min; column temperature, 40°C. Symbols as in Fig. 4.

N values were higher at basic than at acidic pH.

Influence of acetate concentration. Variations of acetate concentration were obtained by dilution of 0.2 M aqueous ammonium acetate solution at pH 6.75 with water. The studied concentrations were 0.2, 0.1, 0.075, 0.050, 0.025 and 0.01 M, each solution containing 0.75% of 1-butanol.

Variations in k', α, R_s, d_0 and N values with acetate concentration are displayed in Fig. 6A–E, respectively. Except for N and R_s, for which some variations occurred for acetate concentration of 0.05 and 0.075 M, all the other parameters were indepen-

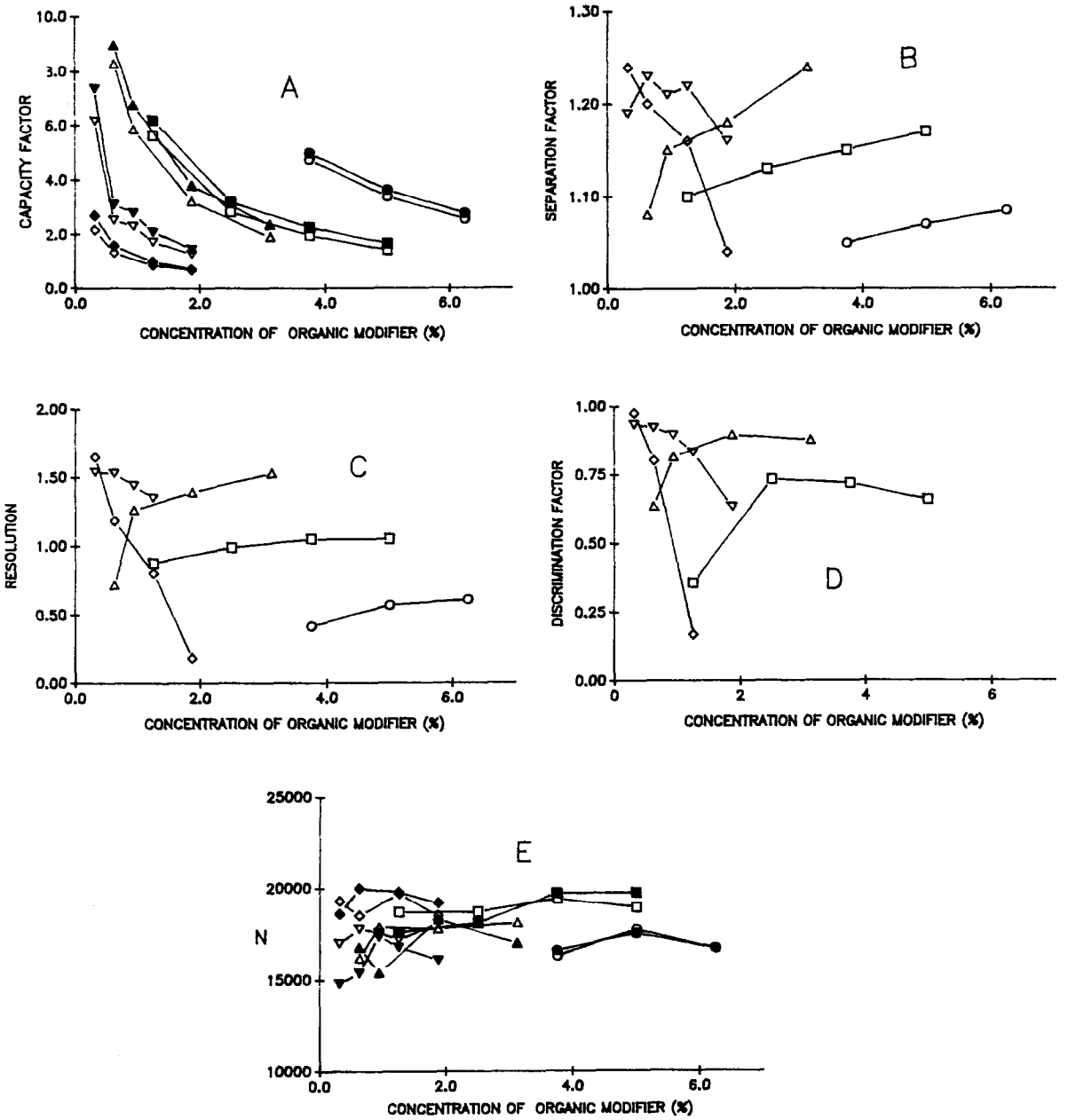

Fig. 7. Acetate buffer: influence of nature and concentration of organic modifier on chromatographic parameters. (A)–(E) as in Fig. 2. Buffer of the mobile phase, 0.1 *M* ammonium acetate (pH 6.75); flow-rate, 1.0 ml/min; column temperature, 40°C. Symbols as in Fig. 4.

dent of the acetate concentration. Because of the partial destruction of the column during the pH study, some results, obtained under the same analytical conditions, were different from those described above; nevertheless, the variations in the chromatographic parameters observed in this study remained meaningful as they were obtained with a column that was still able to separate the two optical isomers.

Influence of organic modifier. The mobile phase

was 0.1 *M* ammonium acetate aqueous solution at pH 6.75 to which various amounts of different organic modifiers were added. In order to have sufficient accuracy, the amount of organic modifiers was weighed and converted to volume using a density value of 0.8. Variations of k' values with organic modifier concentration are displayed in Fig. 7A. The elution order was always the same, CRL 40982 being eluted before CRL 40983 whichever organic solvent was used. The inversion of elution order observed with ethanol in phosphate buffer did not occur with ammonium acetate. In each instance, the difference between k' values of CRL 40982 and CRL 40983 was small, so the extreme α values (Fig. 7B) were 1.04 (1.875% 1-pentanol) and 1.24 (0.3% 1-pentanol). In the range of organic modifier concentrations used in this study, the α values increased in relation to the concentration of ethanol, 1-propanol and 2-propanol, were nearly independent of 1-butanol concentration, and decreased with 1-pentanol concentration.

The R_s values (Fig. 7C) were almost independent of the concentration of ethanol (0.4–0.6) and 2-propanol (0.9–1.05) and increased from 0.72 to 1.53 for 1-propanol and decreased slowly for 1-butanol from 1.54 to 0.88 and rapidly for 1-pentanol from 1.65 to 0.18.

The d_0 values (Fig. 7D) were zero for ethanol at any concentration and for 1-pentanol at 1.875%. For 1- and 2-propanol they reached a maximum (0.9 for 1-propanol at 1.875% and 0.74 for 2-propanol at 2.5%). The d_0 values slowly decreased for 1-butanol (from 0.94 to 0.63) and very rapidly for 1-pentanol (from 0.95 to 0.17).

Regarding variations in the numbers of theoretical plates (Fig. 7E), no trends could be observed. The N values ranged from 15 000 to 20 000 plates/m.

DISCUSSION

Choice of buffer

The amounts of organic modifier used for studying the influence of buffers were derived from preliminary results and were chosen to obtain approximately the same values of k' at neutral pH in each buffer for both CRL 40982 and CRL 40983. The significant variations in chromatographic parameters reported above were principally obtained more for pH modifications than for ionic strength.

Variations in k', indicating variations in thermodynamic equilibrium between the solute and the chromatographic system, were significant only *versus* pH variations in phosphate buffer and could not be used for optimization of the separation as both the compounds were affected in the same way by pH variations. The thermodynamic changes were due not only to pH but also to a more complex phenomenon as they did not occur in acetate buffer in the same range of pH. The influence of pH was more effective on α values: for both the buffers, the linear variation of α with pH indicated a better separation of the two isomers at neutral or basic pH (but basic pH was precluded because of instability of the column in acetate buffer). The effect of pH variations was more important in acetate buffer (slope of linear regression $= 5.82 \cdot 10^{-2}\ k'$ unit per pH unit) than in phosphate buffer (slope of linear regression $= 3.31 \cdot 10^{-2}\ k'$ unit per pH unit).

The ionic strength of the buffer did not exhibit any thermodynamic effect as, for both buffers, no variation in k' with salt concentration was significant. In the same way, the kinetic modifications, showed by variations in R_s, d_0 and N (if the k' values remained constant) due to ionic strength variations were small: only N variations $<25\%$ and R_s variations $<15\%$ were reported.

The kinetic influence of pH was stronger particularly for phosphate buffer: d_0 and R_s increased almost linearly from pH 5 to 7 then remained constant; the d_0 values were increased 2.5-fold between pH 6 and 7 and the R_s values doubled between pH 6 and 7.1. For acetate buffer, only increases in R_s values were really significant, being 1.4-fold between pH 5.55 and 6.75.

Whereas the influence of pH appeared to be weaker in acetate buffer than in phosphate buffer, the absolute values of all the measured chromatographic parameters (for the same k' values) were always larger for acetate buffer than for phosphate buffer. At neutral pH and with the same values of k' and α, in terms of R_s, d_0 and N, the benefit was equal to factors of 2.4, 1.7 and 2.1, respectively. Consequently, ammonium acetate is to be preferred to sodium dihydrogenphosphate as the buffer in order to optimize the separation of modafinil optical isomers.

From a practical point of view, equilibration volumes for Chiral-AGP columns were lower with phosphate buffer (15–20 ml of mobile phase) than with acetate (at least 30 ml), but the lifetime of the column was considerably longer with acetate. In routine work using phosphate buffer as the mobile phase, one needed to add a small amount of Chiral-AGP slurry to the top of the column at least once a week, because of the occurrence of a void volume. Such an occurrence of a void volume had not been observed using acetate buffer, even after 4 months of constant use.

Choice of organic modifier

The results obtained in the study of the influence of organic modifiers confirmed the interest in using ammonium acetate as buffer: for each organic modifier used at the same concentration, values of the chromatographic parameters indicated a better separation using ammonium acetate than sodium dihydrogenphosphate.

To a first approximation, the thermodynamic and kinetic effects of each organic modifier were similar for both buffers. A more detailed examination of the results showed some differences for ethanol which

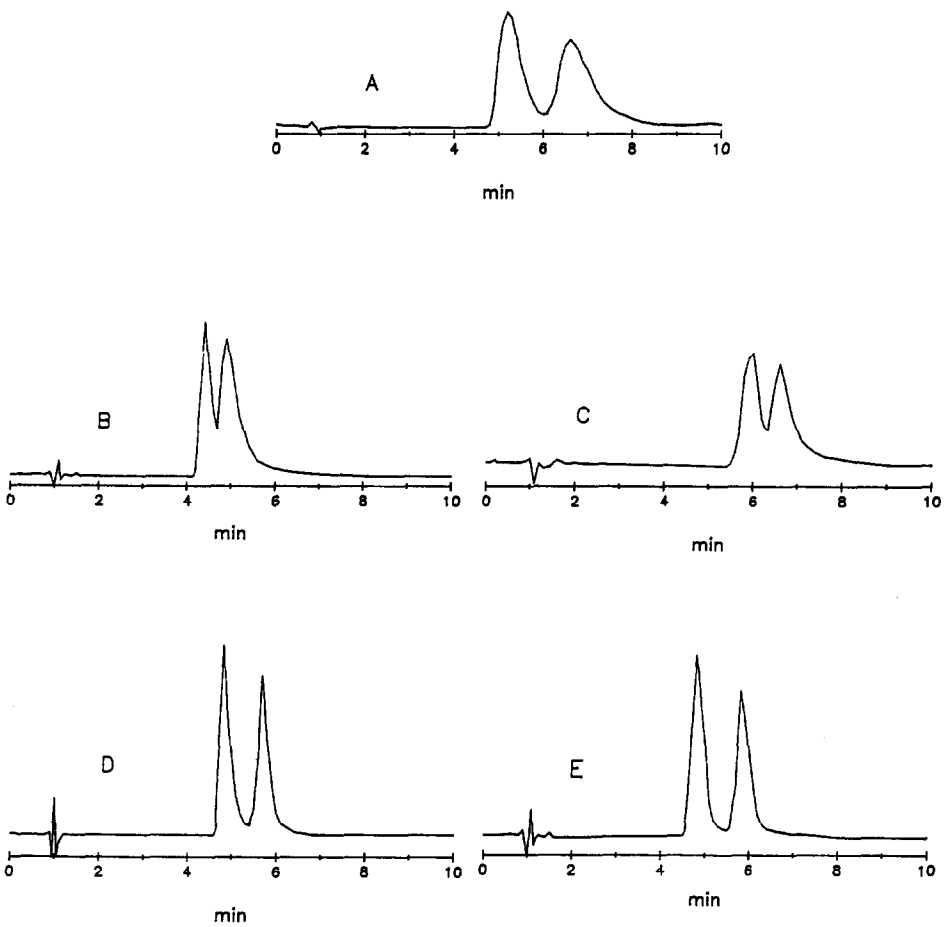

Fig. 8. Chromatograms of Modafinil optical isomers. Chiral AGP column (100 × 4.6 mm I.D.); flow-rate, 1.0 ml/min; temperature, 40°C; detection wavelength, 267 nm [except (A), 220 nm]. (A) 1.5% 1-propanol in 0.03 *M* phosphate buffer (pH 7); (B) 0.5% 1-butanol in 0.02 *M* phosphate buffer (pH 7); (C) 0.15% 1-pentanol in 0.02 *M* phosphate buffer (pH 7); (D) 0.5% 1-butanol in 0.1 *M* acetate buffer (pH 6.75); (E) 0.1% 1-pentanol in 0.1 *M* acetate buffer (pH 6.75).

were probably due to the inversion of the retention order observed for ethanol in phosphate buffer. For both buffers, the eluotropic order was 1-pentanol > 1-butanol > 1-propanol > 2-propanol > ethanol. For acetate buffer the decreasing order of maximum values of α, d_0 and R_s was the same. For phosphate buffer, the order was 1-pentanol > 1-butanol > ethanol > 1-propanol > 2-propanol. Because of drastic variations in the chromatographic parameters with any small variation in the amount of 1-pentanol in the mobile phase, the use of this organic modifier in routine work is not recommended. For this purpose, the use of 1-butanol, for which the consequences of variations were less important, is to be preferred.

CONCLUSIONS

The chromatograms in Fig. 8 highlight the improvement in the separation of modafinil optical isomers obtained after the optimization study of the mobile phase. The use of ammonium acetate as buffer and of 1-butanol as organic modifier allowed real benefits in terms of selectivity, resolution and sensitivity. The use of this kind of mobile phase has already been extended in our laboratory to the separation of other optical isomers, always with the same result: a better resolution and a longer lifetime of the chromatographic column.

Replacement of potassium phosphate buffer by ammonium acetate makes the use of the Chiral AGP column in LC–TSP-MS analysis possible. Consequently, pharmacokinetics studies of Modafinil optical isomers could be considered.

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