Chiral separations of atropine and homatropine on an α_1-acid glycoprotein-bonded stationary phase

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SUMMARY

Chiral separations of cationic compounds were studied with two structurally very similar compounds, atropine and homatropine. Retention, resolution and sensitivity were studied by isocratic and gradient elution techniques using changes in pH, temperature and concentrations of 2-propanol and anionic additives.

Anions of octanoic acid and 2-phenylbutyric acid improve the chiral separation of atropine whereas the chiral selectivity for homatropine can be suppressed. (*R*)- and (*S*)-2-phenylbutyric acid give different separation factors for atropine, which indicates a specific interaction with the solute. The antipodes of the cations are retained by different mechanisms in the presence of anionic additives, as demonstrated by experiments with factorial design, Van 't Hoff plots and the indirect detection technique.

INTRODUCTION

Enantiomeric separations are of considerable practical importance, *e.g.*, in the development of new drugs and new therapy in the biomedical field. Direct liquid chromatographic separations using chiral stationary phases have many advantages and the number of such phases is increasing. Most of them have a narrow field of application. However, phases that contain α_1-acid glycoprotein as the chiral selector have a unique position in this respect as they are suitable for separations of charged and uncharged enantiomers with widely different structures. The wide application is due in part to the fact that the enantioselectivity can be varied and adapted to different solutes by additives to the mobile phase[1].

EnantioPac was the first commercial chiral phase containing α_1-acid glycoprotein[2], but a new generation of this material, Chiral-AGP, with a partical size of 5 μm and improved chromatographic performance, has been developed[3]. The properties and

applications of EnantioPac have been reported in numerous papers, part of them summarized in some recent reviews[1,4]. α_1-Acid glycoprotein contains several chiral centres, but the mechanism for the chiral binding is so far unknown. However, some empirical conclusions on structural requirements for chiral binding have been presented[1]. The separations are performed with an aqueous phase and retention and selectivity can be changed by varying the pH, uncharged, cationic and anionic additives and temperature. The effect of these changes on the chromatographic conditions is highly dependent on the structure of the solute and even small structural differences can have large effects. A drastic example is the widely different influences of charged additives on the chromatographic behaviour of methylatropine and methylhomatropine[5]

The aim of this study was to develop methods for the separation of cationic enantiomers on the Chiral-AGP phase and to optimize the systems with respect to retention, stereoselectivity and sensitivity. Atropine and homatropine were used as test samples. They have very small differences in structures (Fig. 1), atropine having one methylene group more than homatropine in a substituent at the chiral centre. However, changes in the chromatographic conditions often have different effects on their chromatographic behaviour on Chiral-AGP.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of an LKB (Bromma, Sweden), Model 2150 pump, a Rheodyne 7010 injector with a 20-μl loop and a Spectroflow 783 variable-wavelength detector (Kratos, Ramsey, NJ, U.S.A.). The column, injector and connecting tubes were thermostated using an RM6 (Messgeräte-Werk Lauda, Lauda-Königshofen, F.R.G.) or a Thermomix 1441 (Braun Melsungen, Melsungen, F.R.G.). The chromatograms were recorded on a Perkin-Elmer 56 instrument. The gradient studies were performed with two LKB Model 2150 pumps controlled by a Model 2152 HPLC controller.

Chemicals

Atropine sulphate, homatropine bromide and sodium octanoate were purchased from Merck (Darmstadt, F.R.G.), hyoscyamine, the *S*-enantiomer of atropine, from Boehringer (Mannheim, F.R.G.), (*R*)- and (*S*)-2-phenylbutyric acid from Sigma (St. Louis, MO, U.S.A.) and 4-phenylbutyric and 4-pentylbenzoic acid from Fluka (Buchs, Switzerland).

Fig. 1. Structures of atropine and homatropine.

Chromatographic conditions

The separation column was a Chiral-AGP (100 × 4.0 mm I.D.; 5 μm) from ChromTech (Stockholm, Sweden). The flow-rate used was 0.4 ml/min and the system was thermostated at 22°C if not stated otherwise. The mobile phases were phosphate buffers (ionic strength, $\mu = 0.05$) to which modifiers were added. The mobile phases for the gradient systems differed only in their content of 2-propanol and the gradient profiles were linear. The dead volume of the gradient system was 3 ml, which was taken into account by making the sample injection coincide with the gradient reaching the column inlet. The wavelength of detection was the UV absorption maximum of the solute, if not stated otherwise.

RESULTS AND DISCUSSION

Isocratic regulation of retention

pH and charged and uncharged modifiers have a large influence on retention and selectivity, as previously reported. The magnitude, however, is strongly dependent on the structure of the solute.

The effects of pH, 2-propanol and sodium octanoate on retention and selectivity were studied using a factorial design[6]. The factorial design gives information about whether a probable variable really is affecting the response, to what extent and whether the response is dependent on a combination of variables. An advantage with factorial design is that as much information as possible is achieved from a limited number of experiments. In a complete factorial design with three variables where all possible combinations at the two levels are investigated, eight (2^3) experiments are made.

The compositions of the mobile phases used are summarized in Table I.

The model describing the response (retention or chiral selectivity) can be expressed as

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 +$$

$$+ \beta_{123} x_1 x_2 x_3 + \varepsilon \quad (1)$$

TABLE I

FACTORIAL DESIGN, REGULATION OF RETENTION

Expt. No.	$x_1{}^a$	$x_2{}^b$	$x_3{}^c$
1	−1	−1	−1
2	+1	−1	−1
3	−1	+1	−1
4	−1	−1	+1
5	+1	+1	−1
6	+1	−1	+1
7	−1	+1	+1
8	+1	+1	+1

[a] x_1 = pH in the mobile phase: 6.0 (−1) or 7.5 (+1).
[b] x_2 = Concentration of 2-propanol in the mobile phase: 0% (−1) or 3% (+1).
[c] x_3 = Concentration of octanoate in the mobile phase: 0 mM (−1) or 4 mM (+1).

TABLE II

CALCULATED EFFECTS (β-COEFFICIENTS) OF pH (x_1), 2-PROPANOL (x_2) AND OCTA-
NOATE (x_3) FOR ATROPINE

Parameter	β_1	β_2	β_3	β_{12}	β_{13}	β_{23}
k'_1	6.36	−5.84	−0.62	−4.44	0.09	0.52
k'_2	8.86	−8.30	1.80	−6.70	2.20	−1.54
α	0.06	−0.10	0.19	−0.04	0.04	−0.08

where β_n describes the effect of the variable x_n and β_{mn} correspondingly describes the
combined effect of the variables x_m and x_n. The β-coefficients were calculated by
multiplying the level of each variable with its response, adding the products and
dividing the sum by the number of experiments.

The calculated effects are summarized in Table II for atropine and in Table III
for homatropine. The calculated effects show for both solutes that an increased pH
gives increased retention whereas the selectivity is almost unaffected. An increased
content of 2-propanol in the mobile phase leads to a lower retention and selectivity.
Similar effects of pH and addition of an uncharged modifier have been reported
before and are probably due to increased negative charge of the protein and competi-
tion for the binding sites, respectively. The β-coefficients for octanoate show that it
affects the retention less than pH and 2-propanol. The retention of the first-eluted
enantiomer decreases with increasing octanoate concentration for both atropine and
homatropine. For atropine the retention of the second-eluted enantiomer is increased
by an increasing concentration of octanoate, giving an increased chiral separation.
For homatropine the effect is the opposite, an increase in octanoate concentration
decreases both k'_2 and the stereoselectivity.

The combined effects were generally smaller than the individual effects. How-
ever, the combined effect of pH and 2-propanol affects the retention negatively, less
than pH and 2-propanol individually but more than octanoate.

Gradient elution

The effects of different mobile phase additives under isocratic conditions can be
applied to improve the detection sensitivity using gradient elution.

The effect of the concentration of 2-propanol in the mobile phase on the reten-
tion (k') and resolution (R_s) of homatropine is demonstrated in Fig. 2. With an
increase in the concentration of 2-propanol the retention and resolution decrease
whereas the sensitivity increases owing to a reduction in peak width. The correspond-

TABLE III

CALCULATED EFFECTS (β-COEFFICIENTS) OF pH (x_1), 2-PROPANOL (x_2) AND OCTA-
NOATE (x_3) FOR HOMATROPINE

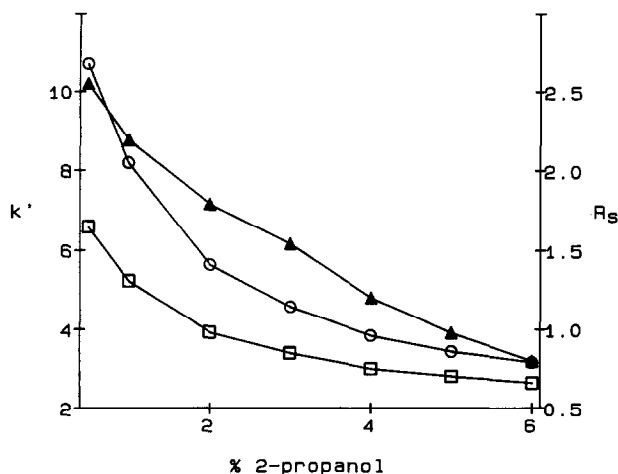| Parameter	β_1	β_2	β_3	β_{12}	β_{13}	β_{23}
k'_1	4.96	−3.51	−0.10	−2.67	0.08	−4.20
k'_2	7.09	−5.40	−1.17	−4.06	−0.66	1.05
α	0.04	−0.09	−0.14	0.01	0.02	−0.005

Fig. 2. Isocratic elution with 2-propanol as modifier. Solid phase: Chiral-AGP. Mobile phase: phosphate buffer (pH 7.0) + 2-propanol. Detection wavelength: 254 nm. Solute: racemate of homatropine. $\square$ = k'_1; $\bigcirc$ = k'_2; $\blacktriangle$ = R_s.

ing effect of 2-propanol on the retention and resolution of the atropine enantiomers is demonstrated in Fig. 3. In this instance sodium octanoate was present in the mobile phase in order to obtain a sufficient stereoselectivity.

The detection sensitivity and resolution are both dependent on the steepness of the gradient in solvent gradient elution. The sensitivity increases and the resolution decreases with a steeper gradient, assuming a linear solvent strength gradient. Studies on Chiral-AGP with metoprolol showed that the decrease in retention times is balanced by the peak compression effect when the gradient is moderately steep, resulting
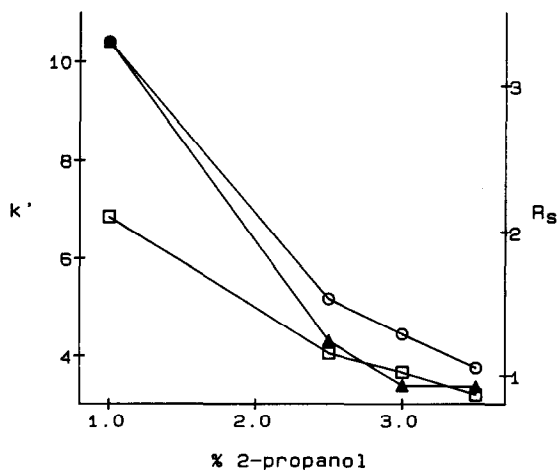
Fig. 3. Isocratic elution with 2-propanol as modifier. Solid phase: Chiral-AGP. Mobile phase: phosphate buffer (pH 7.0) + 2.5 mM octanoate and 2-propanol. Detection wavelength: 220 nm. Solute: racemate of atropine. $\square$ = k'_1; $\bigcirc$ = k'_2; $\blacktriangle$ = R_s.

TABLE IV

OPTIMIZATION OF SENSITIVITY AND RESOLUTION USING ISOCRATIC AND GRADIENT
ELUTION TECHNIQUES

Mobile phase: phosphate buffer (pH 7.0) + 2-propanol (IPA). Solute: homatropine. Detection wavelength: 254 nm.

Start IPA (%)	Δ IPA (%/min)	h_1/m^a	h_2/m^a	R_s
0.5	0	1.00	0.69	2.55
6.0	0	2.59	2.46	0.80
0.5	0.37	1.98	1.88	1.76
0.5	2.2	2.30	2.31	1.54
0.5	5.5	2.50	2.35	1.49

[a] Relative sensitivity.

in almost unchanged resolution[7]. It was further observed that in a linear solvent strength gradient the sensitivity was the same for all the solutes eluted under gradient conditions, in accordance with the relationships given by Snyder et al.[8].

Table IV shows gradient elution effects on homatropine. By using a steep gradient elution almost the same sensitivity is achieved as when using isocratic elution with a high concentration of 2-propanol. However, the gradient technique gives a baseline resolution (R_s = 1.5) whereas with isocratic elution the resolution is not complete (R_s = 0.8).

For atropine the gradient elution effects had to be studied with octanoate present in the mobile phase (Table V). The highest sensitivity is obtained isocratically with a high content of 2-propanol but the resolution is incomplete. A complete resolution is obtained by gradient elution but the value of the gradient technique is doubtful. It gives a certain decrease in the sensitivity and no improvement for the second enantiomer as the peak-height ratio h_2/h_1 is the same as in the isocratic run. The advantage of the propanol gradient seems to be lost when the retention is influenced by octanoate.

TABLE V

OPTIMIZATION OF SENSITIVITY AND RESOLUTION USING ISOCRATIC AND GRADIENT
ELUTION TECHNIQUES

Mobile phase: phosphate buffer (pH 7.0) + 2.5 mM octanoate + 2-propanol (IPA). Solute: atropine. Detection wavelength: 220 nm.

Start IPA (%)	Δ IPA (%/min)	h_1/m^a	h_2/m^a	R_s
0.8	0	1.00	0.63	2.61
2.5	0	2.17	1.70	1.31
0.8	0.11	1.98	1.54	1.88

[a] Relative sensitivity.

Effect of temperature

Temperature can also be used as a tool to regulate retention and resolution (see Table VI). The highest sensitivity is achieved using isocratic elution with a high concentration of 2-propanol and high temperature (No. 4 in Table VI). Comparison between the use of a 2-propanol gradient and isocratic elution at low temperature (Nos. 5 and 1) shows that the peak-height ratios for the two enantiomers are $h_{1,grad.}/h_{1,iso.} = 1.84$ and $h_{2,grad.}/h_{2,iso.} = 2.45$, *i.e.*, the peak heights are doubled when using a gradient. The ratio between the resolutions is only 10% lower using the gradient technique ($R_{s,grad.}/R_{s,iso.} = 0.90$). In the example given above, gradient elution is not necessary, as isocratic elution (No. 4) gives the highest sensitivity with baseline resolution. However, the gradient technique can be useful for optimizing the chromatographic systems when the resolution is lower.

The influence of temperature can be illustrated by Van 't Hoff plots, where ln k' is plotted *versus* the reciprocal of absolute temperature. A straight line indicates a single retention mechanism[9]. Plots for atropine are given in Fig. 4. Two mobile phases were used, both having the same pH and content of 2-propanol but one also containing octanoate. The octanoate has a very significant effect on the stereoselectivity and in its absence a very poor resolution is obtained.

The plots for the first-eluted enantiomer of atropine, hyoscyamine, with and without octanoate are similar, indicating that octanoate is only slightly involved in the retention of hyoscyamine. The last-eluted enantiomer has higher slope and intercept in the presence of octanoate.

The corresponding plots for homatropine are given in Fig. 5. The slope and intercept of the first-eluted enantiomer are also here of the same magnitude with and without octanoate. Addition of octanoate decreases the slope and intercept for the second-eluted enantiomer of homatropine, which means that the effect is the opposite to that obtained with atropine. This is in accordance with the results given in Tables II and III. The β-coefficients for octanoate are positive for atropine and negative for homatropine. The reason might be that the second-eluted enantiomer of atropine deviates by being retained as an ion pair with octanoate.

TABLE VI

INFLUENCE OF TEMPERATURE (T) AND CONCENTRATION OF 2-PROPANOL ON RESOLUTION AND SENSITIVITY

Mobile phase: phosphate buffer (pH 7.0) + 2-propanol (IPA). Solute: homatropine. Detection wavelength: 254 nm.

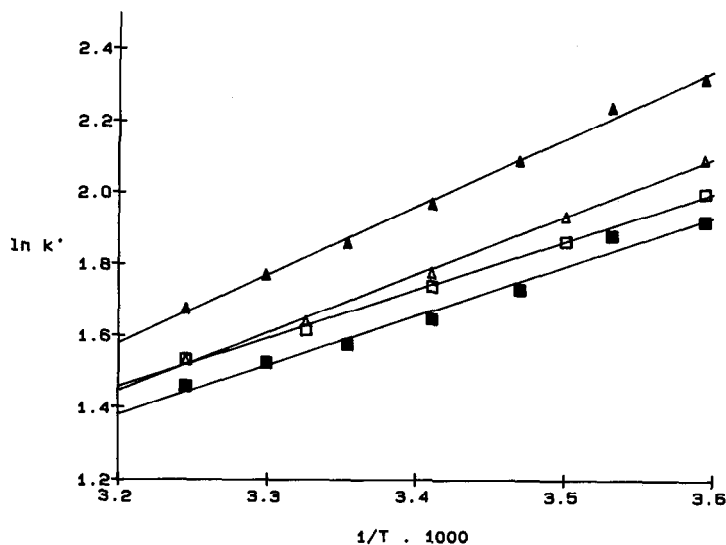| No.	Start IPA (%)	Δ IPA (%/min)	Temp. (°C)	h_1/m^a (°C/min)	h_2/m^a	R_s
1	0.6	—	5	1.00	0.57	3.54
2	2.5	—	5	2.26	1.38	3.01
3	0.6	—	35	2.70	1.96	2.18
4	2.5	—	35	4.79	3.79	1.49
5	0.6	0.13	5	1.84	1.38	3.17
6	0.6	0.13	35	3.29	2.76	2.08

[a] Relative sensitivity.

Fig. 4. Van 't Hoff plots for atropine. Mobile phase: filled symbols, phosphate buffer (pH 7.0) + 2.5 mM octanoate and 2% 2-propanol; open symbols, phosphate buffer (pH 7.0) + 2% 2-propanol. $\square$,$\blacksquare$ = k'_1; $\triangle$,$\blacktriangle$ = k'_2.

Effects of anionic additives

Improvement of sensitivity. It has been shown in this and in previous studies[10] that a hydrophobic anion such as octanoate is essential for the enantioselectivity of atropine on an α_1-acid glycoprotein bonded phase. This additive can also improve the sensitivity by indirect detection effects.
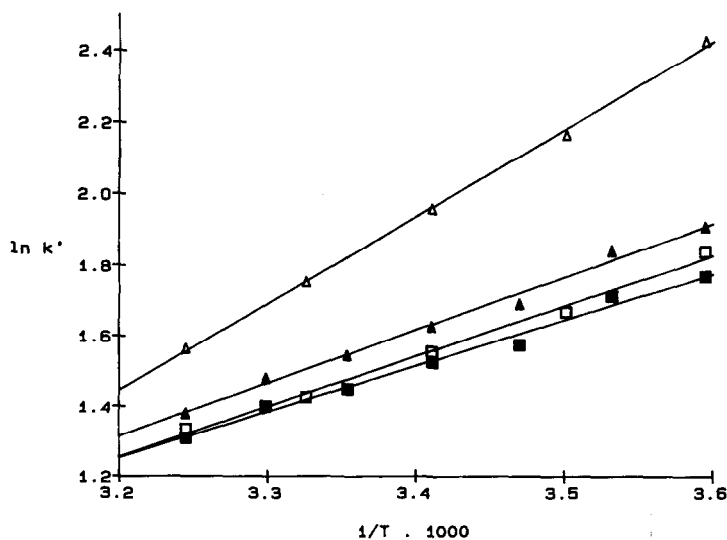
Fig. 5. Van 't Hoff plots for homatropine. Mobile phase and symbols as in Fig. 4.

Indirect detection effects appear when the mobile phase contains a detectable component (a probe) and the probe and injected solute have a common interaction. Injection of any solution differing in composition from the mobile phase, *e.g.*, a solute dissolved in the mobile phase, will disturb the established equilibria and zones with a deficit or excess of the probe will pass through the column. The resulting chromatogram will hence contain positive and negative peaks originating from injected solutes and mobile phase components (system peaks). A positive indirect response effect can improve the sensitivity for a solute with low detector response[11]. A cationic solute, such as atropine, will in a reversed-phase system get a positive indirect response if it is more strongly retained than an anionic probe. The probe will then give rise to a negative system peak. Studies in systems with a hydrophobic adsorbent as the solid phase have shown that the indirect response depends on the retention of the solute peak relative to the system peak and on the fractional loading of the probe on the adsorbent[11]. It should be emphasized that the expression for the relative response given in that study is valid for an adsorbing solid phase and may not be directly applied to a solid phase such as Chiral-AGP with a complex and unknown retention mechanism.

Octanoate has a fairly high molar absorptivity at low wavelengths (210–220 nm). The apparent molar absorptivities, ε^*, of the enantiomers of atropine calculated from the peak area[12] are given in Table VII. They are equal to that obtained for atropine in the absence of an anionic additive. Both enantiomers had similar apparent molar absorptivities. Changes in the concentration of octanoate or the organic modifier 2-propanol had no significant influence on the response. This indicates that the loading of the anionic probe on the AGP phase does not have the effect on the response that is predicted by expressions valid for a hydrophobic adsorbent[11]. The response pattern was normal with a negative system peak appearing before the positive solute peak, indicating a common interaction between solute and probe.

Four anions with high molar absorptivity were tested for their effects on the response and the enantioselectivity on the AGP phase. 4-Pentylbenzoic acid and 4-phenylbutyric acid gave low chiral selectivity and incomplete separation of the enantiomers (Table VII). Both anions gave positive and indistinct system peaks before the solute peak, *i.e.*, no indication of a common interaction with the solute.

TABLE VII

INFLUENCE OF ANIONIC MODIFIERS ON THE DETECTION SENSITIVITY OF ATROPINE

Mobile phase: 1% 2-propanol + anionic modifier in phosphate buffer. Detection wavelength: 220 nm.

Modifier	Concentration $\times 10^4$ (M)	pH	k'_1	α	R_s	ε_1^{*b}	ε_2^{*b}
Octanoate	25	7.0	7.0	1.52	4.0	3500	3500
(S)-2-phenylbutyrate	5	6.0	2.9	3.34	3.7	4700	5800
4-Phenylbutyrate	5	6.0	2.1	1.20	0.85	–	–
4-Pentylbenzoate	5	6.0	6.0	1.06	0.3	–	–
–[a]	–	6.0	2.2	–	–	3400	

[a] Injection of (S)-atropine (hyoscyamine).
[b] ε_1^*, ε_2^* = Apparent molar absorptivities of first- and second-eluted enantiomers, respectively.

(S)-2-Phenylbutyric acid gave high chiral selectivity and improved sensitivity (Table VII). The response pattern contained negative system peaks and positive peaks for both enantiomers (Fig. 6). The apparent molar absorptivity of both eluted enantiomers is higher than that obtained in the absence of an anionic probe. The first peak gave a smaller ε^* than the second peak, contrary to the response obtained in systems with an adsorbing solid phase. This might indicate that the two enantiomers have different retention mechanisms.

Influence on retention. (S)-2-Phenylbutyric acid gives a greater improvement in the enantioselectivity for atropine than octanoate, as shown in Table VII. The separation factor for atropine increases with increasing concentration of (S)-2-phenylbutyric acid (Fig. 7), mainly due to an increase in k' of the second-eluted enantiomer (Fig. 8). The k' increase might indicate binding of the solutes as an ion pair[13] and, as the anion has a chiral centre, the diastereomeric ion pair can be bound on both a chiral and an achiral uncharged site.

The first-eluted enantiomer, which is (S)-atropine (hyoscyamine), is only slightly affected by an increase in the anion concentration. This might be due to binding as an ion pair combined with ionic binding to a charged site on the AGP phase, which has a negative charge in the pH range used[14].

(S)- and (R)-2-phenylbutyric acid give different retentions and chiral selectivities (Table VIII), which indicates that the interaction between the enantiomers of atropine and the chiral anion has a specific character. The first-eluted enantiomer has the same retention with an R- and an S-form of the anion at pH 7.0, which supports the assumption of ionic binding to a charged site. At pH 6.0 the first-eluted enantiomer has a higher retention when (S)-2-phenylbutyric acid is used as an anionic additive. This might be due to the fact that the AGP phase is less strongly charged at that pH, which leads to a less strong dominance of ionic binding over ion-pair distribution.
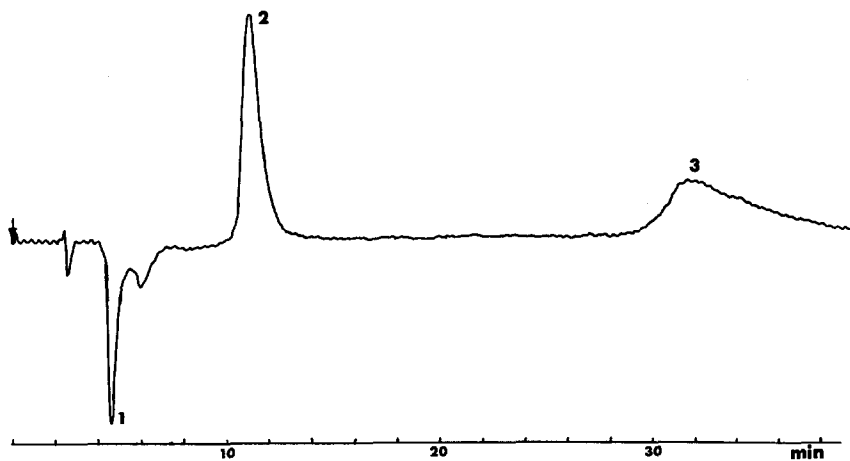

Fig. 6. Resolution of atropine enantiomers. Mobile phase: $5 \cdot 10^{-4}$ M (S)-2-phenylbutyric acid + 1% 2-propanol in phosphate buffer (pH 6.0). Detection wavelength: 220 nm. Peaks: 1 = system peak; 2, 3 = solute peaks.
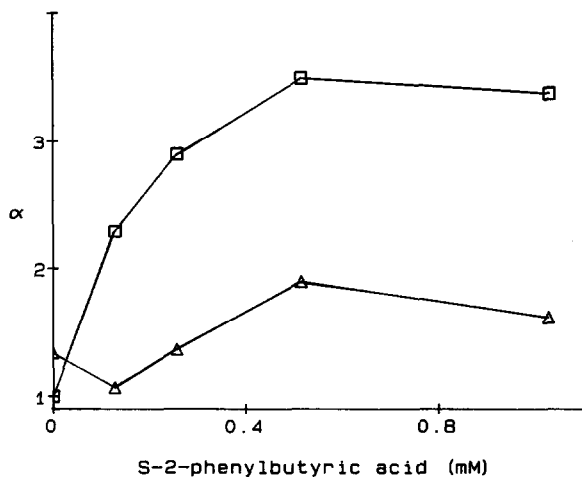
Fig. 7. Influence of (S)-2-phenylbutyric acid on separation factors. Mobile phase: (S)-2-phenylbutyric acid + 1% 2-propanol in phosphate buffer (pH 6.0). Detection wavelength: 220 nm. □ = Atropine; △ = homatropine.

The separation factor of homatropine also increases with increasing concentration of (S)-2-phenylbutyric acid (Fig. 7). The chiral selectivity is much lower and depends almost completely on an increase in k' of the last eluted enantiomer (Fig. 8).

Octanoate as an anionic additive gives a considerably smaller separation factor for atropine than (S)-2-phenylbutyric acid, even though it is used at higher concentration. However, the column efficiency and the peak symmetry are considerably better with octanoate and the resolution is the same as or better than that obtained with (S)-2-phenylbutyric acid (Table VII).
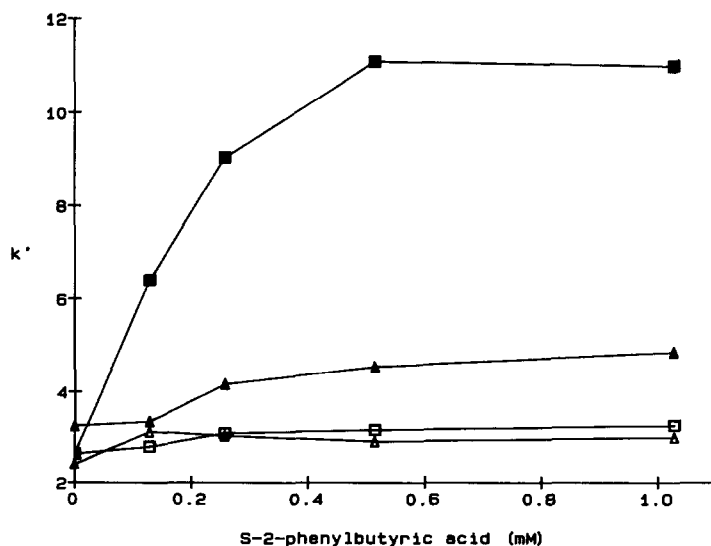

Fig. 8. Influence of (S)-2-phenylbutyric acid on capacity factors. Mobile phase, detection wavelength and symbols as in Fig. 7. △,□ = k'_1; ▲,■ = k'_2.

TABLE VIII

EFFECT OF ANTIPODES OF 2-PHENYLBUTYRIC ACID ON CHIRAL SEPARATION OF ATROPINE

Mobile phase: $5 \cdot 10^{-4}$ M antipode of 2-phenylbutyric acid + 2-propanol in phosphate buffer. Detection wavelength: 258 nm.

Antipode of modifier	2-Propanol (%)	pH	k'_1	α	R_s
S	1	6.0	3.57	3.42	3.8
R	1	6.0	2.57	1.59	1.9
S	2	7.0	4.50	1.44	1.7
R	2	7.0	4.50	1.25	1.1

For homatropine, the retention of both enantiomers decreases with increasing concentration of octanoate. The second-eluted enantiomer is more strongly affected than the first, leading to a decrease in chiral selectivity at higher octanoate concentration.

CONCLUSION

Atropine and homatropine are structurally very similar but they show large differences in chromatographic behaviour on Chiral-AGP, particularly in the presence of anions of octanoic acid and 2-phenylbutyric acid. The anionic additives also have different influences on the antipodes of the solutes. The differences indicate binding by sites or mechanisms of different character, which in part can be due to the fact that the binding sites can be charged or uncharged. The different separation factors obtained for atropine with the enantiomers of 2-phenylbutyric acid indicate a specific steric interaction between anion and cation which might occur in the mobile phase or on the solid phase.

SYMBOLS

h/m sensitivity (peak height/moles solute injected);
R_s resolution $[2\Delta t_R/(W_1 + W_2)]$;
α separation factor (k'_2/k'_1);
k' capacity factor $[(t_R - t_0)/t_0]$;
t_0 time for elution of a front peak, obtained by injecting a solution diverging from the mobile phase, *e.g.*, water.
t_R retention time;
W peak width at the base, obtained by tangents

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