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OPTIMIZATION OF DETECTION SENSITIVITY FOR ENANTIOMERS OF METOPROLOL ON SILICA-BONDED α_1-ACID GLYCOPROTEIN

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SUMMARY

On the chiral phases CHIRAL-AGP and EnantioPac, organic modifiers, pH and temperature have been varied with isocratic and gradient techniques to obtain minimum retention and maximum peak height by baseline resolution of *R*-and *S*-metoprolol. The decrease in retention obtained by gradient elution gives, by limiting baseline resolution, 20–60% greater peak heights than those obtained by the isocratic technique. A reduction of the retention by a change in the pH is more favourable than addition of modifiers such as acetonitrile or 2-propanol. It gives a smaller decrease in resolution by isocratic elution and a pH gradient can give considerably stronger peak compression than an acetonitrile gradient as indicated by an estimation of the detection limits. For metoprolol enantiomers, CHIRAL-AGP gives about three times lower reduced plate heights than does EnantioPac.

INTRODUCTION

Most drugs with chiral properties are used as racemic mixtures although in many cases the desired physiological effect is due mainly to one of the enantiomers. The pharmacokinetics can also be different for the antipodes and it is therefore important separately to investigate each form in order to estimate the therapeutic effect of administration of a racemic mixture[1].

Liquid chromatography is the technique employed most often for the resolution of enantiomers, and differences in retention are achieved by interaction of the solutes with another enantiomeric molecule, a chiral selector, which can be coupled to the solutes by covalent or coordinative bonds. The enantiomers of a 1,2-aminoalcohol like metoprolol can thus be separated as derivatives with 2,3,4,6-tetra-O-acetyl-β-D-glycopyranosyl isothiocyanate[2], with a complexing selector such as N-benzoxycarbonylglycyl-L-proline (ZGP) present in the mobile phase[3] and with a stationary phase containing a chiral selector such as α_1-acid glycoprotein (AGP)[4] or cellulose tris-3,5-dimethylphenyl carbamate[5].

The determination of metoprolol in biological samples requires an high mass

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sensitivity. Low concentrations can be measured by fluorimetry[6], but the enantiomer peaks must be eluted in small volumes and with sufficient resolution. The choice of the chromatographic separation conditions is of great importance since it has been observed that many liquid chromatographic systems for chiral separation give rather wide peaks.

The present study was performed with silica-bonded AGP as the chiral phase. Two commercial phases were used, EnantioPac and CHIRAL-AGP, the latter being a recent development with improved separating efficiency and stability. The aim of the investigation was to develop chromatographic conditions for high detection sensitivity by complete resolution of the antipodes of metoprolol.

EXPERIMENTAL

Apparatus

The chromatograph comprised a Model 2150 pump with a Model 2152 gradient controller (LKB, Bromma, Sweden), a WISP 710 B autosampler (Waters Assoc., Milford, MA, U.S.A.) and a fluorescence detector LS4 (Perkin-Elmer, Norwalk, CT, U.S.A.), which was set at 228 nm (excitation) and 306 (emission), with slit widths of 10 nm. Chromatograms were recorded using a Model 4270 integrator (Spectra-Physics, San José, CA, U.S.A.).

The separation columns were EnantioPac, 100 mm × 4.0 mm, 10 μm (LKB) and CHIRAL-AGP, 100 mm × 4.0 mm, 5 μm (ChromTech, Stockholm, Sweden). The CHIRAL-AGP column was protected by a Brownlee DIOL guard column in a module (Brownlee Labs., Santa Clara, CA, U.S.A.). The temperature of the columns was controlled by a thermostat bath RMS 6 (Lauda, Königshofen, F.R.G.). The pH of the eluent was measured in a flow cell, constructed at AB Hässle, using a glass electrode GK 743500 and a Model 84 pH-meter (Radiometer, Copenhagen, Denmark) with a Perkin-Elmer 56 recorder (Hitachi, Tokyo, Japan).

Chemicals and reagents

The racemate and the separate enantiomers of metoprolol and α-hydroxymetoprolol were synthesized at the Department of Organic Chemistry, Hässle. 2-Propanol was of high-performance liquid chromatography (HPLC) grade and acetonitrile of specially pure HPLC grade S (Rathburn Chemicals, Walkerburn, U.K.). All other reagents and buffer substances were of analytical grade (E. Merck, Darmstadt, F.R.G.). Water used in the mobile phase was from a Milli-Q system (Millipore, Molsheim, France).

Chromatographic system

The mobile phase comprised phosphate buffer (I = 0.02) modified with 2-propanol or acetonitrile in isocratic or gradient mode. In the pH gradient system the mobile phase contained 0.5% 2-propanol in phosphate buffer pH 7.5 (I = 0.02) (a) and 0.02 M sodium dihydrogenphosphate (b) in various proportions. The gradient pumping system had a volume of 2.0 ml from the mixing chamber to the column inlet, which was taken into account by making the sample injection coincide with the gradient reaching the column inlet. The solutes were injected dissolved in the mobile phase, if not stated otherwise. The volumes injected were 10 or 20 μl.

The flow-rate of the mobile phase was 0.3 ml/min for the EnantioPac and 0.5 ml/min for the CHIRAL-AGP column. The experiments were performed on a number of columns. The efficiency of the columns, expressed as the theoretical plate numbers, decreased after some time and many injections, but the separation factor, α, was almost constant. Reactivating the column by a low flow of 25% 2-propanol in water or 0.01 *M* H_3PO_4 was sometimes successful. The separate experimental series presented in a table or a figure were performed without change of the column.

Calculations

The parameters, used in the evaluation of the column and separation efficiency are the plate number, N, reduced plate height, h, α and resolution, R_s

$$N = 5.54(t_R/W_{\frac{1}{2}})^2$$

$$h = \text{column length}/(N \cdot \text{particle diameter})$$

$$\alpha = k'_S/k'_R$$

$$R_s = 2(t_{R_s} - t_{R_R})/(W_R + W_S)$$

where t_R = retention time in min, $W_{\frac{1}{2}}$ = width at the half peak height in min, W_R. W_S = widths of the peaks at the base in min and $k' = (t_R - t_0)/t_0$. The determination of t_0, *i.e.*, the retention time of a non-retained compound, was performed by injecting the hydrophilic α-hydroxymetoprolol. The content of 2-propanol in the mobile phase was increased gradually until no decrease of the retention time for α-hydroxymetoprolol was noticed. The value of t_0 was 4.2 min (1.26 ml) for the EnantioPac column and 2.2 min (1.10 ml) for the CHIRAL-AGP column in this system.

RESULTS AND DISCUSSION

Properties of α_1-acid glycoprotein and the AGP columns

AGP is an human transport protein. Its molecular weight is about 41 000 and its isoelectric point is 2.7 in phosphate buffer. It contains a peptide chain with 181 amino acid units and five carbohydrate units, the latter constituting 45% of the molecular weight. There are numerous asymmetric centres in the peptide chain and the carbohydrate units but the chiral binding principle has so far not been elucidated[7].

The chiral stationary phase based on AGP was developed by Hermansson[8] and its properties and applications have been presented in a large number of publications (*cf.*, ref. 9). The commercial product EnantioPac contains the protein bound to diethylaminoethylsilica and immobilized by cross-linking. Eluents outside the range pH 4–7 should be avoided. CHIRAL-AGP is a newly developed product with improved chromatographic properties and stability. The useful range is up to pH 7.5 and temperatures between 3 and 40°C can be used.

The two AGP phases can be applied to chiral separations of moderately hydrophobic compounds having charged or hydrogen-bonding groups in the vicinity of the chiral centre. The retention and the chiral selectivity can be changed by uncharged modifiers in the aqueous mobile phase such as acetonitrile and lower

alcohols. Changes in the pH and temperature as well as the addition of charged modifiers are other means for regulation of retention and chiral selectivity[9].

Isocratic regulation of retention

The influence of two uncharged modifiers, acetonitrile and 2-propanol, on the retention of *R*-metoprolol and the chiral separation factor, α, on an EnantioPac column is demonstrated in Figs. 1 and 2. 2-Propanol has a strong effect even at concentrations below 1% while acetonitrile must be used in about five times higher concentrations to give the same decrease of k'_R. With both modifiers the decrease in the retention is accompanied by a rather limited decrease in the separation factor. The strong influence of the modifiers on the retention indicates an interaction with the AGP phase that is more specific than a simple competition for an hydrophobic binding site (*cf.*, ref. 9).

The effect of temperature changes is highly dependent on the structure of the solute[4,10]. An increase from 7 to 28°C decreases k' of *R*-metoprolol by about 20% on CHIRAL-AGP (Table I). On EnantioPac the temperature effect on k' seems to be somewhat larger, possibly due to a different binding of AGP to the silica matrix.

AGP has an isoelectric point of 2.7 and the negative charge and the binding ability for cationic solutes increase with pH, but the effect is dependent on the structure of the solute[4,10–12]. On CHIRAL-AGP the k' of *R*-metoprolol decreases to one third upon a decrease in pH of slightly more than one pH unit (Table II). This strong decrease in retention has, however, a very limited effect on the chiral selectivity. A comparison with other means for regulation of the retention indicates that a pH change that causes a similar change in k' has a considerably lower influence on the

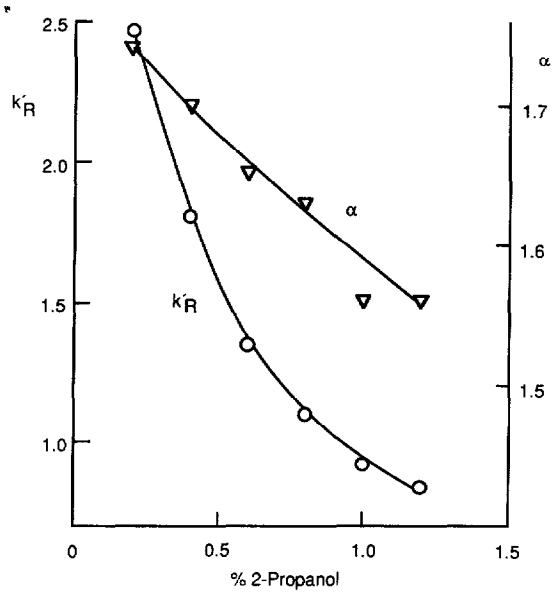

Fig. 1. Isocratic elution with 2-propanol as a modifier. Solid phase: EnantioPac. Temperature: 12°C. Mobile phase: phosphate buffer pH 7.0 with 2-propanol. Solutes: *R*- and *S*-metoprolol.

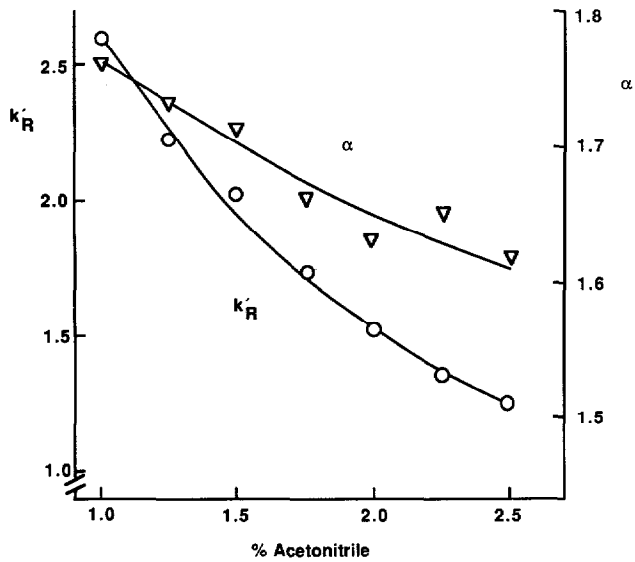

Fig. 2. Isocratic elution with acetonitrile as a modifier. Conditions as in Fig. 1, except: mobile phase, phosphate buffer pH 7.0 with acetonitrile.

chiral selectivity than changes in the modifier concentration or temperature, which might open possibilities to elucidate the nature of the chiral binding sites on AGP.

The phases with silica-bonded AGP give high chiral selectivity for compounds of widely different structures. In order to obtain chiral resolution this selectivity must be combined with good separating efficiency which is counteracted by many chiral solid phases giving rather wide solute peaks. The background to the low efficiency has not so far been explained but speculations on slow binding due to conformational changes have been presented[12]. Studies on EnantioPac have shown that the peak width is dependent on the structure of the solute, and that the temperature and the composition of the mobile phase can also have an influence[10,12].

CHIRAL-AGP gives better reduced plate heights than EnantioPac. Illustrations of the differences in separating efficiency are given in Tables I and III. Acetonitrile is

TABLE I

TEMPERATURE EFFECTS BY ISOCRATIC ELUTION

Solid phase: CHIRAL-AGP. Mobile phase: phosphate buffer pH 7.0 with 1.65% acetonitrile.

Temp. (°C)	k'_R	α	h_R	h_S	R_s
7	5.76	1.44	12	11	3.3
10	5.48	1.43	11	10	3.4
13	5.24	1.42	11	10	3.3
16	5.10	1.38	10	9	3.1
19	4.95	1.37	12	9	3.1
22	4.76	1.34	9	8	3.0
25	4.62	1.32	9	8	2.8
28	4.48	1.30	9	8	2.6

TABLE II

pH EFFECTS BY ISOCRATIC ELUTION

Solid phase: CHIRAL-AGP. Mobile phase: phosphate buffer with 0.5% 2-propanol. Temperature: 20°C.

pH	k'_R	α	R_s
7.50	7.67	1.53	3.6
7.29	6.69	1.54	3.2
7.08	4.82	1.52	2.9
6.91	4.25	1.51	2.8
6.69	3.30	1.49	2.4
6.40	2.53	1.45	2.0
6.02	1.42	1.38	–

the modifier in both experimental series. Table I shows the effect of temperature variation at constant modifier concentration on CHIRAL-AGP, whereas Table III gives the results obtained with EnantioPac when the modifier concentration is varied at constant temperature (12°C). CHIRAL-AGP gives about three times lower h values than does EnantioPac and the resolution, R_s is much higher despite the fact that the separation factor, α, on CHIRAL-AGP is lower under similar chromatographic conditions.

Improvement of detection sensitivity by chromatograhic means

The aim of the study was to develop chromatographic conditions that give complete resolution of the enantiomers and minimize the volumes in which the peaks are eluted.

The detection sensitivity can be improved by decreasing the retention until a limiting baseline resolution ($R_s = 1.5$ for symmetrical peaks) is obtained. On the AGP columns this can be performed in a systematic way by changing the modifier concentration, pH or temperature as indicated above. Application of the retention changing agent in a gradient mode is a convenient and rapid way of reaching the limiting conditions. Furthermore, if the principles for linear solvent strength gradients can be applied to this system, gradient elution should give better detection sensitivity than the corresponding isocratic elution when the resolution is the same[13,14].

TABLE III

ISOCRATIC ELUTION WITH ACETONITRILE AS THE MODIFIER

Solid phase: EnantioPac. Mobile phase: phosphate buffer pH 7.0 with acetonitrile. Temperature: 12°C.

Acetonitrile (%)	α	h_R	h_S	R_s
1.00	1.76	26	22	1.9
1.25	1.73	28	25	1.6
1.50	1.71	30	27	1.4
1.75	1.66	35	26	1.3
2.00	1.63	37	24	1.2
2.25	1.65	40	31	1.1
2.50	1.62	33	26	1.1

The evaluation of the sensitivity in gradient elution can be based on the peak width at the base, $W_b = 4\sigma_t$, or the signal per mol of solute at a given R_s. (Band widths in gradient elution can not be used for evaluation of the separating efficiency.) With a linear solvent strength gradient the sensitivity, s_g, is according to Snyder[13] given by

$$s_g = 1/G(1 + k_f) \quad (1)$$

where $k_f = 1/2.3b$ corresponds to k' of a solute band leaving the column in gradient elution, b expresses the steepness of the gradient and G is a band compression factor which is roughly equal to 0.8 at a moderate value of b.

The detection sensitivity, s_g, is improved by increasing the gradient steepness, *i.e.*, $1/b$ has an effect analogous to k' in isocratic liquid chromatography. Eqn. 1 shows furthermore that the sensitivity should be the same for all solutes independent of their retention. This means, *e.g.*, that the enantiomers in a racemate should give peaks of equal height.

Further means for improvement of detection sensitivity such as injection of large volumes of sample and use of microbore or capillary columns have not been included in this study.

Gradient elution with organic modifier

The resolution in gradient elution can be described by an expression analogous to that used for isocratic elution. Snyder[13] has shown that k' in the expression for R_s can be substituted for $\bar{k} = 1/1.3b$ when a linear solvent strength gradient is used. If other chromatographic conditions are unchanged, R_s will then be proportional to $[\bar{k}/(1 + \bar{k})]^2 = [1/(1 + 1.3b)]^2$. An increase in the gradient steepness, b, will obviously decrease R_s whereas the detection sensitivity is increased as shown above.

A comparison of the detection sensitivity by isocratic and gradient separation on CHIRAL-AGP with acetonitrile as the modifier is given in Fig. 3. Both elution modes have been adjusted to give the same resolution, $R_s = 2.0$. The amounts of *R*- and *S*-metoprolol injected are also the same, 36 pmol of each. The gradient elution gives two peaks of about equal height and the peak height quotients for the two enantiomers are $R_{grad}/R_{isocr} = 1.20$ and $S_{grad}/S_{isocr} = 1.62$, *i.e.*, 20 and 62% higher sensitivity for the gradient elution.

The expression for the influence of gradient steepness on detection sensitivity and resolution given above has been evaluated for a linear solvent strength gradient

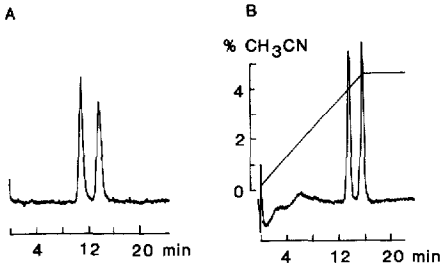

Fig. 3. Separation of metoprolol enantiomers. Solid phase: CHIRAL-AGP. Temperature: 20°C. Amount injected: 36 pmol of each. Mobile phase: phosphate buffer pH 7.0 with (A) isocratic elution and 2.25%. acetonitrile; (B) gradient elution, starting with 0.1% acetonitrile, gradient 0.293%/min.

TABLE IV

ELUTION WITH 2-PROPANOL GRADIENT

Solid phase: EnantioPac. Mobile phase: phosphate buffer pH 7.0 with 2-propanol. Temperature: 3°C. Solutes: *R*- and *S*-metoprolol, 30 pmol of each.

2-Propanol		*Rel. peak height*		R_s
Start (%)	*Gradient (%/min)*	*R*	*S*	
0.24	–	1	0.70	1.5
0.2	0.010	1.08	0.86	1.5
0.2	0.015	1.16	0.97	1.5
0.1	0.020	1.19	1.14	1.5
0.1	0.027	1.19	1.14	1.4
0.04	0.048	1.35	1.35	1.1
0.04	0.064	1.54	1.54	1.1

and an adsorbing stationary phase. A qualitative test of its validity for the separation of metoprolol enantiomers on EnantioPac with 2-propanol as the modifier is presented in Table IV. EnantioPac gives an higher chiral selectivity than that of CHIRAL-AGP (see Tables I and III) but the separating efficiency with isocratic elution is much lower. Isocratic elution with 0.24% 2-propanol gives an almost complete resolution. Gradient elution with a steepness up to 0.02%/min gives no significant loss of resolution but the detection sensitivity is improved. The enantiomer peaks have about equal height and the increase of the detection sensitivity is 20% for the *R*-enantiomer and about 60% for the *S*-enantiomer. A steeper gradient gives a slight decrease in R_s but the sensitivity improvement is substantial: more than 50% for the *R*-enantiomer and more than 100% for the *S*-enantiomer.

The gradient studies show that the peak compression effects outbalance the decrease in retention difference between the enantiomers when the gradients are moderately steep. This indicates that the steepness of the modifier gradient on the AGP phase has a less strong influence on the resolution than indicated by the expression for R_s given by Snyder[13]. Gradient elution seems to be of special value as a tool for improvement of the detection sensitivity on the AGP phase.

Elution with pH gradients

The strong effect of pH on the retention of metoprolol on CHIRAL-AGP was shown in Table II. A further indication of the importance of pH is given by the chromatographic results shown in Fig. 4. The solutes are normally injected with mobile phase as the solvent and chromatogram A was obtained in that way. Chromatogram B was obtained with the same amount of solutes dissolved in 0.01 *M* H_3PO_4. The first peak (*R*-metoprolol) has the same width on both chromatograms, whereas the second peak (*S*-metoprolol) is significantly narrower in chromatogram B. Measurement of the pH in a flow cell inserted after the column showed that the phosphoric acid injected (10 µl) had caused a pH pulse that migrated with the same speed as that of *S*-metoprolol.

The pH effect on the retention can be used in gradient elution. The slow migration of the pH pulse shown in Fig. 4B indicates that the hydrophilic components

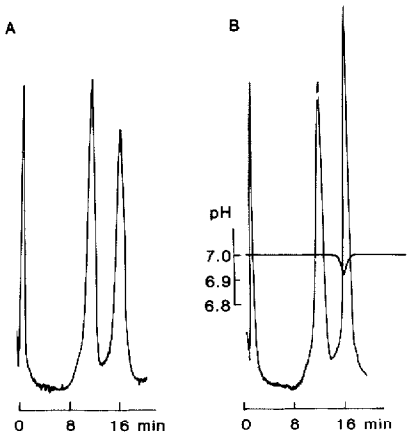

Fig. 4. Influence of sample solvent. Solid phase: EnantioPac. Temperature: 3°C. Mobile phase: phosphate buffer pH 7.0 with 1.5% acetonitrile. Solutes: *R*- and *S*-metoprolol. Sample solvents: (A) mobile phase; (B) 0.01 *M* H_3PO_4.

in the buffer will be retained. The effect of the pH gradient will depend on its migration relative to the solutes and the shape and the retention of the gradient must be determined experimentally. An example is given in Fig. 5. The gradient was obtained from phosphate buffer pH 7.5 and 0.02 *M* sodium dihydrogenphosphate and was applied to a CHIRAL-AGP column. The pH was measured in flow cells at the inlet and the end of the column. The gradient has the same shape before and after the column in the range pH 6.3–7.3 where the phosphate buffer has its highest capacity.

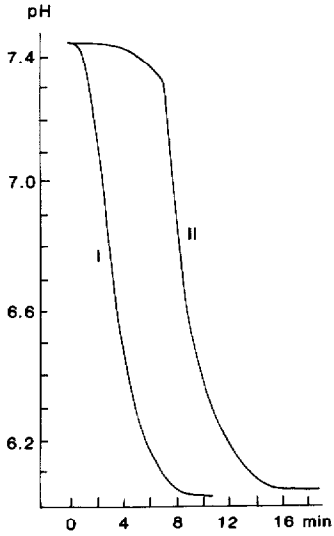

Fig. 5. Effect of the pH gradient. Solid phase: CHIRAL-AGP. Temperature: 20°C. Mobile phase: 0.5% 2-propanol in phosphate buffer pH 7.5 (I = 0.02) (a), 0.02 *M* sodium dihydrogenphosphate (b). Start: pH 7.4 (98% a + 2% b). Gradient: linear increase of b from 2 to 75% during 1 min. End: pH 6.05 (25% a + 75% b). pH measured in flow cells: I, before column; II, after column.

The deviations at lower buffer capacity might be due to the measuring technique. The retention and the shape of the gradient are highly dependent on the nature of the buffer components as has been found by studies of, *e.g.*, citrate buffers.

The application of pH gradients to the separation of *R*- and *S*-metoprolol on EnantioPac is shown in Fig. 6. The gradients covered the pH range 7.3–6.2 and the solutes were injected at such a time that the pH change mainly affected the last eluted *S*-enantiomer. The compression of the peak depends on the steepness of the gradient and the steepest gradient (Fig. 6C) increases the peak height of the *S*-enantiomer by a factor of about three compared to the isocratic elution (Fig. 6A). When the sample was injected so that both enantiomers were affected by the pH change incomplete separation was achieved.

CHIRAL-AGP has a considerably higher separating efficiency than EnantioPac and it is easy to apply a pH gradient in such a way that the detection sensitivity of both metoprolol enantiomers is improved with the maintenance of complete resolution. Some examples are given in Fig. 7. Isocratic separation at pH 7.5 with 0.5% 2-propanol as a modifier gives a separation with $R_s = 3.3$ (Fig. 7A). The high resolution gives excellent possibilities of application of pH gradients, and the effect of gradients with different steepness covering the range pH 7.5–6.0 are shown in Fig. 7C and D. The peak height improvements by gradient elution relative to the isocratic elution at pH 7.5 are summarized in Table V.

Fig. 7D shows a limiting baseline resolution obtained by the pH-gradient technique. The limiting resolution in isocratic elution obtained by pH adjustment is shown in Fig. 7B. The improvement of the detection sensitivity relative to isocratic elution at pH 7.5 is considerable as shown in Table V. However, gradient elution to the same limiting resolution gives a further improvement of 50–60%.

An estimation from Fig. 7D gives a detection limit, defined as three times the noise, of about 0.7 pmol of each enantiomer. The acetonitrile gradient in Fig. 3B gives an estimated detection limit of about 1.8 pmol. This indicates that the pH gradient

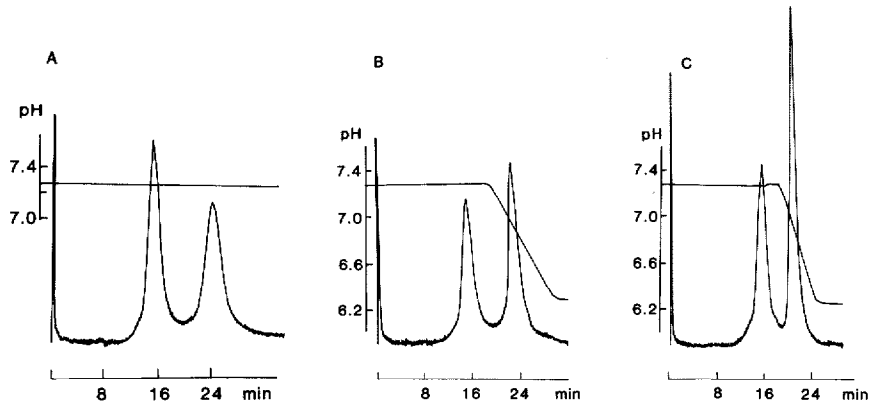

Fig. 6. Elution with a pH gradient. Solid phase: EnantioPac. Temperature: 5°C. Solutes: *R*- and *S*-metoprolol, 90 pmol of each. Mobile phase: see Fig. 5. (A) Isocratic, pH 7.3. (B) Start: pH 7.3 (96% a + 4% b). Gradient: linear increase of b from 4 to 75% during 25 min. End: pH 6.2 (25% a + 75% b). (C) pH as in (B), gradient time 1 min (preceded by isocratic elution for 4 min). The pH after the column is given.

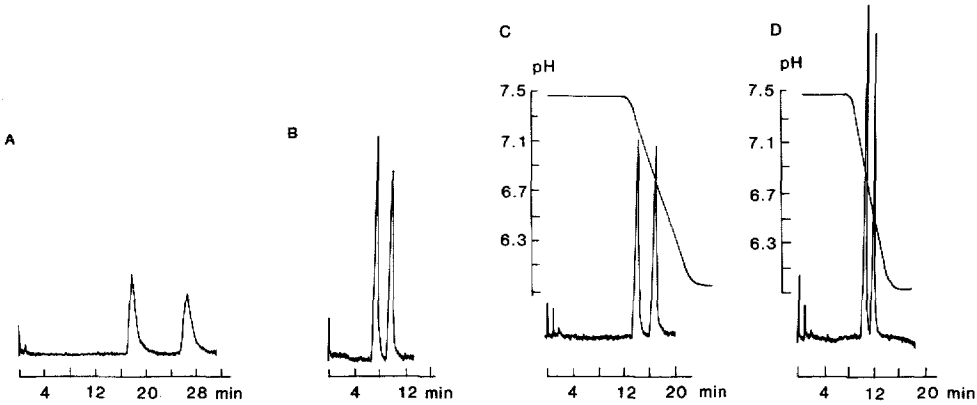

Fig. 7. Influence of pH by isocratic and gradient elution. Solid phase: CHIRAL-AGP. Temperature: 20°C. Solutes: *R*- and *S*-metoprolol, 18 pmol of each. Mobile phase: see Fig. 5. (A) Isocratic: pH 7.50. (B) Isocratic: pH 6.40. (C) Start: pH 7.5 (100% a). Gradient: linear increase of b from 0 to 75% during 15 min. End: pH 6.0 (25% a + 75% b). (D) pH as in (C), gradient time 1 min. The pH after the column is given.

gives a stronger peak compression than the acetonitrile gradient but quantitative conclusions can be made only if differences in the column properties, if any, are taken into consideration.

Improvement of detection sensitivity on AGP phases

The AGP phases can be used for separation of enantiomers of widely different structures but the influence on the chiral selectivity of modifiers, pH and temperature can be highly different even for structurally closely related substances[9,12]. It is important to remember that pH changes have different effects on the retention of cationic, anionic and uncharged substances. Certain enantiomers can be separated only in the presence of charged modifiers and addition of a modifier can sometimes even give rise to a reversal of the retention order between the solutes[4,9]. Preliminary studies of the effects of changes of the chromatographic conditions must be made before optimization of the sensitivity is attempted.

TABLE V

DETECTION SENSITIVITY BY ISOCRATIC AND GRADIENT ELUTION

Solid phase: CHIRAL-AGP. Mobile phase: phosphate buffer with 0.5% 2-propanol. Temperature: 20°C. Solutes: *R*- and *S*-metoprolol, 18 pmol of each.

pH		*Relative peak height*	
Start	*ΔpH/min*[a]	*R*	*S*
7.50	–	1	0.75
6.40	–	2.8	2.3
7.5	−0.1	2.5	2.4
7.5	−1.5	4.3	3.8

[a] Before the column.

The aim of the optimization should be the attainment of baseline resolution at the lowest possible retention by the gradient or isocratic technique. Modifier or pH gradients are tested with increasing steepness until limiting baseline resolution is obtained. Studies by the isocratic technique can be started at a low temperature with the initial aim of finding a pH modifier level that gives $R_s > 1.5$. The limiting baseline resolution can then be reached by increasing the temperature.

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