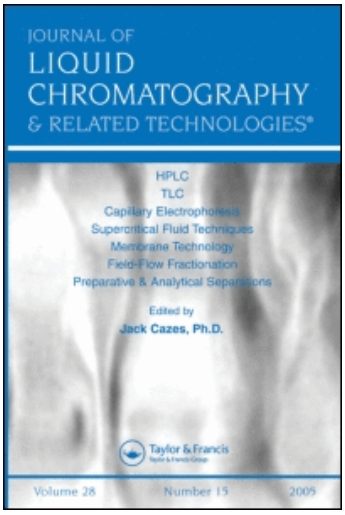


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# Cellulosic Chiral Stationary Phase Under Reversed-Phase Condition

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# CELLULOSIC CHIRAL STATIONARY PHASE UNDER REVERSED-PHASE CONDITION

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ABSTRACT

Chiral stationary phases on polysaccharide esters have been mainly applied under a normal phase condition. However, there are some samples that can not be analyzed under normal phase conditions because of the solubility and the procedures by which they are prepared. We have established reversed-phase conditions of mobile phases to attain good chiral separations on cellulose-based-columns. A simple mixture of water and an organic solvent as the mobile phase gave sufficient separation of an electrically neutral racemate. On the other hand, it was necessary to add an anionic chaotrope for the separation of a basic racemate and a small amount of a strong acid for an acidic one.

## INTRODUCTION

The polysaccharide-based chiral stationary phases we developed[1-a,b,c] have been utilized mainly under normal phase conditions, because their usefulness under reversed-phase conditions had not

been well documented. Recently, papers in which such separations are described were published. The report by Ikeda et al. is particularly important[2]. They attained a good chiral separation of several racemates including propranolol using buffers as the mobile phase on CHIRALCEL OD — a cellulose tris(3,5-dimethylphenylcarbamate)-based column[1-d]. Though they optimized the separation by changing the type of buffer and the pH of the mobile phase, they did not explain their observation. Therefore, we conducted a thorough study of the practicality of the polysaccharide-based-phases under the reversed-phase condition, mainly using CHIRALCEL OD-R.



## MATERIALS

### Chemicals

Acetonitrile (HPLC grade) was purchased from Nacalai Tesque or Wako Pure Chemical, methanol from Nacalai Tesque, sodium perchlorate from Kanto Chemical, perchloric acid (70%) from Nakarai Chemicals, potassium hexafluorophosphate from Tokyo Chemical, sodium hexafluorophosphate from Aldrich, sodium tetrafluoroborate from Tokyo Chemical, and so on. Water was purified with Milli-Q reagent water system (MILLIPORE) or distillation and filtration.

### Instrumentation

A chromatograph consisted of the following (All are products of JASCO.) : a 880-PU pump, a 870-UV detector, a 802-SC system controller, a 880-50 degasser and a 851-AS autosampler. Column temperature was controlled by soaking a column in water thermocontrolled with CTP-100 cooling thermo pump (EYELA, TOKYO RIKAKIKAI). The data obtained were processed and

recorded with CDS86 data system (Nihon-Chromato Works). The columns used were CHIRALCEL OD-R (based on cellulose tris(3,5-dimethylphenylcarbamate)) and CHIRALCEL OJ (based on cellulose tris(4-methylbenzoate)). Both are 25cm in length and 0.46cm in diameter, and are available from Daicel Chemical Industries Ltd.

### Chromatography condition

The conditions are as follows unless otherwise noted : the flow rate was 0.5ml/min, the column temperature was 25°C, and the detecting wavelength was 254nm.



## RESULTS AND DISCUSSION

### Active constitute of a perchlorate buffer

Ikeda et al. obtained the best separation with a perchlorate buffer[2]. However, the system acts a very poor "buffer". We , therefore, wanted to determine the active factor in this "buffer" system that attected the separation.

Figure 1 shows chromatograms of three compounds, each electrically neutral, acidic or basic, with four kinds of mobile phases. They are aq. acetonitrile : one containing sodium perchlorate, one containing perchloric acid, and one containing both ("buffer" solution), respectively.

Indapamide, a neutral compound, was well separated with any of the mobile phases. However, propranolol, a basic compound required a high concentration of perchlorate ion ; N-benzyloxycarbonyl (hereafter abbreviated as CBZ) valine required a small amount of perchloric acid. To detaermine the underlying behavior of the compounds of each group, the following detailed studies were conducted.

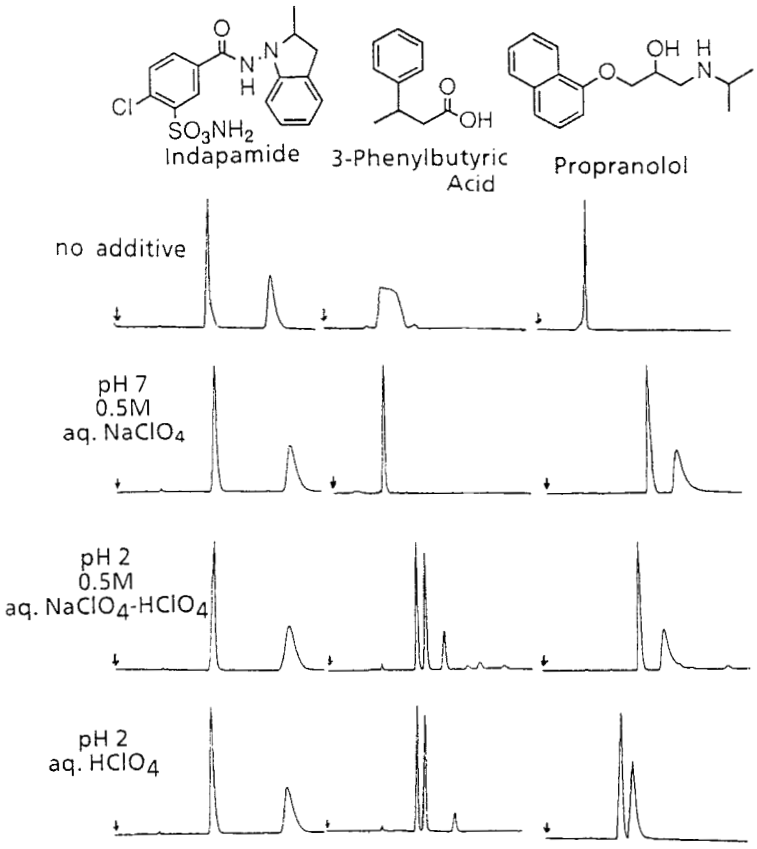


Figure 1.Chromatograms of neutral (indapamide), acidic (3-phenylbutyric acid) and basic (propranolol) compounds with four kinds of mobile phases. They are aq. acetonitrile : one containing sodium perchlorate, one containing perchloric acid, and one containing both, respectively, on a CHIRALCEL OD-R column. mobile phase : aq./$CH_3CN$ = 60/40(v/v). flow rate : 0.5ml/min. column temperature : 25°C. detection : UV 254nm.



## Neutral compounds.

Figure 2 shows the chiral separation of benzoine, 1-phenoxy-2-propanol, and 4-phenyl-2-butanol with the unbuffered mobile phase. The separations were not affected by the addition of perchlorate salt or perchloric acid as well as indapamide. Based on

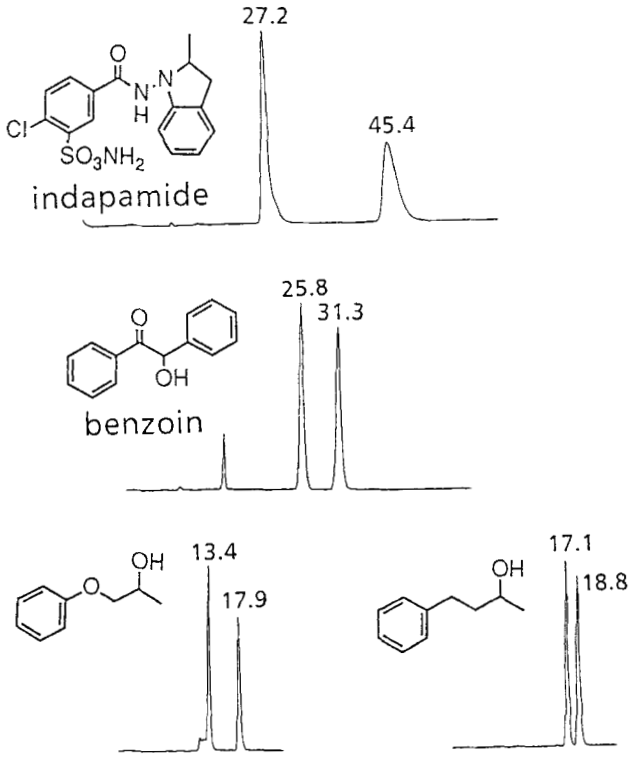


Figure 2. Chromatograms of neutral compounds, indapamide, benzoin, 1-phenoxy-2-propanol, and 4-phenyl-2-butanol on a CHIRALCEL OD-R column. The retention times are given with the numbers near each peak in minutes. mobile phase : $H_2O/CH_3CN = 60/40(v/v)$. flow rate : 0.5ml/ min. column temperature : 25°C. detection : UV 254nm.

these results, we concluded that the chromatographic behavior of a neutral analyte is essentially insensitive to electrolyte addition, and a simple mixture of water and an organic solvent works sufficiently.

Acidic compounds.

As was already refereneced, CBZ-valine and 2-phenylbutyric acid were retained and separated into the enantiomers with an acidic mobile phase. We therefore examined the pH dependence of the

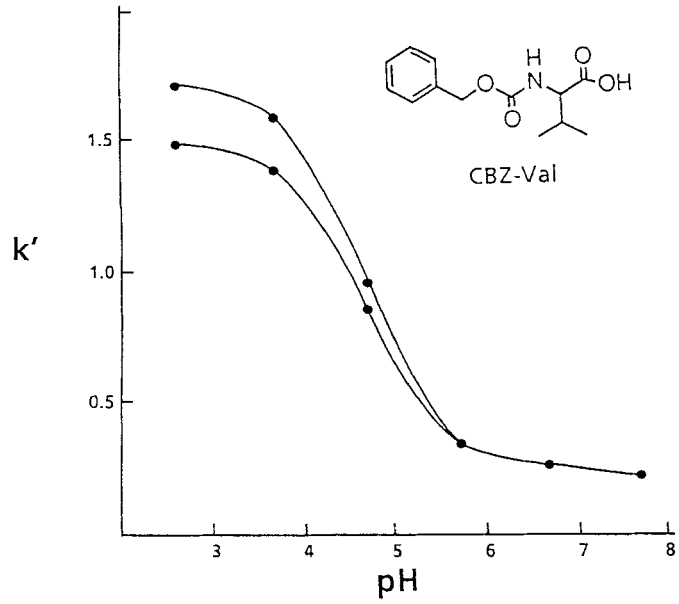


Figure 3. Effect of the pH of the mobile phase on the relative retention of CBZ-Val enantiomers on a CHIRALCEL OD-R column.
mobile phase : aq. phosphate buffer(0.1M)/$CH_3CN$ = 60 /40(v/v). flow rate : 0.5ml/min. column temperature : 25°C. detection : UV 254nm.



retention of CBZ-valine enantiomers using mixtures of phosphate buffers (pH 2~7) and acetonitrile (Figure 3). The plot showed an S-shaped curve, which is characteristic of ionization controlled system[3]. Satisfactory retention and chiral separation were attained only at a low pH (pH < 3). We conclude that the supression of ionization of analyte carboxyl group with a sufficiently acidic mobile phase is necessary to attain a sufficient retention and eventual chiral saparation. In order to clarify what acid to choose, we examined the effect of the addition of perchloric acid and acetic acid (both in suitable amounts to make the pH of the buffer solution at 2.0). As is shown in Figure 4, while perchloric acid was as effective as phosphoric acid, acetic acid reduced the retention. We

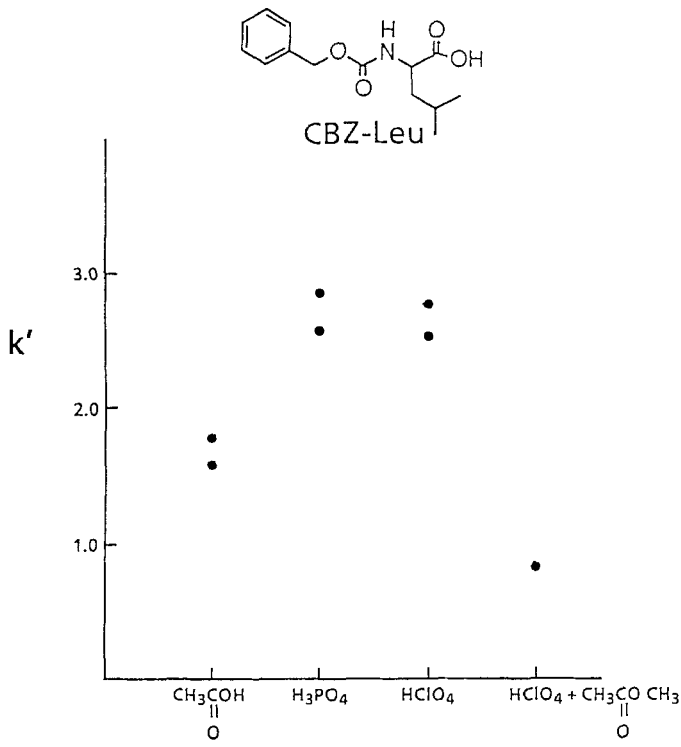


Figure 4. Relative retention of CBZ-Leu enantiomers with mobile phases containing the following acids, $CH_3CO_2H$, $H_3PO_4$, $HClO_4$, and $HClO_4$ with $CH_3CO_2CH_3$ on a CHIRALCEL OD-R column.
mobile phase : pH 2 aq. aqueous acid/$CH_3CN$ = 60/40(v/v). flow rate : 0.5ml/min. column temperature : 25°C. detection : UV 254nm.

speculate that the considerable amount of undissociated acetic acid competitively supressed the retention of the analyte as well as neutral methyl acetate. From this result, addition of a small amount of a strong acid seems suitable for the purpose of pH adjustment. Some other examples of the chiral separation of acidic analytes are given in Figure 5.

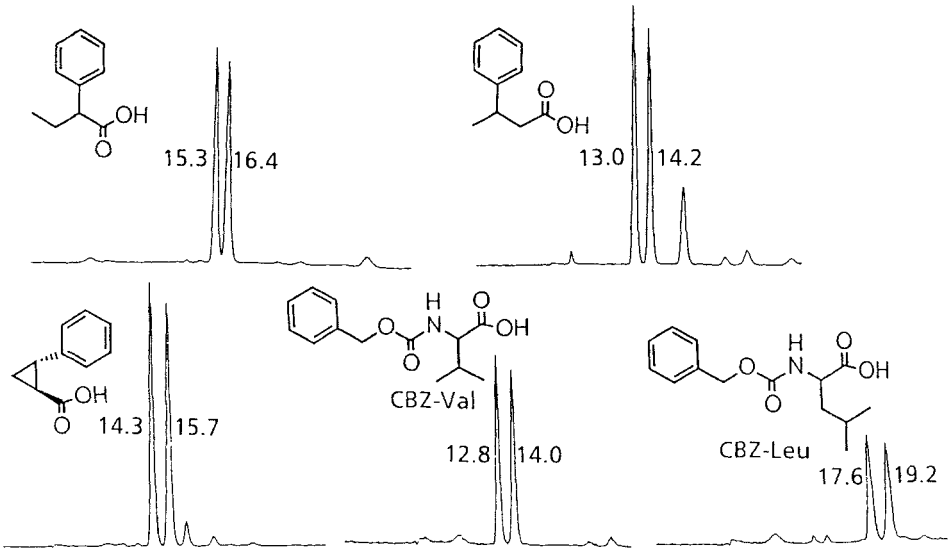


Figure 5. Chromatograms acidic compounds, 2-phenylbutyric acid, 3-phenylbutyric acid, trans-2-phenylcyclopropanecarboxylic acid, CBZ-Val and CBZ-Leu on a CHIRALCEL OD-R column. The retention times are given with the numbers near each peak in minutes.
mobile phase : pH 2 0.5M aq. $NaClO_4$-$HClO_4$/$CH_3CN$ = 60/40(v/v). flow rate : 0.5ml/min. column temperature : 25°C. detection : UV 254nm.



Basic compounds.

The successful resolution of propranolol with a perchloric acid buffer as the mobile phase had been attained by Ikeda et al. as previously mentioned. We first hypothesized that the acidity of the mobile phase would be the essential factor. But, despite our expections, a mobile phase of the same pH containing only perchloric acid gave less retention as shown in Figure 1. Therefore, we examined a mobile phase containing sodium perchlorate and found that it is the constituent contributing to chiral separation (Figure 1). Based on this result, we examined the addition of various salts into the mobile phase. The effect of various anions on the

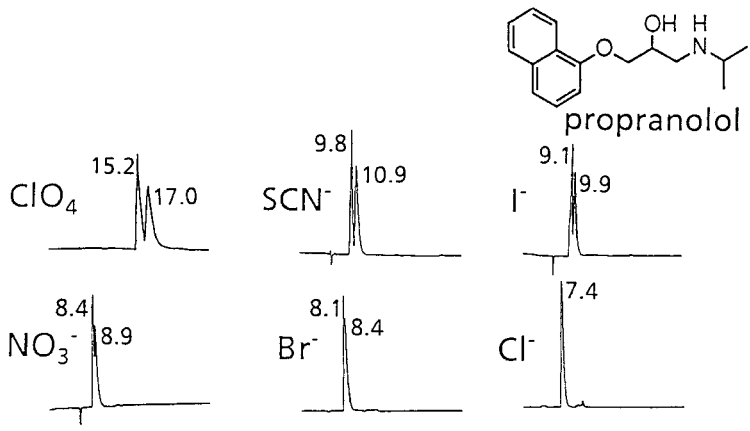


Figure 6. Effect of some chaotropic anions as moible phase additive on the separation of propranolol on a CHIRALCEL OD-R column. The retention times are given with the numbers near each peak in minutes.
mobile phase : 0.1M aq. $Na^+$ salt/$CH_3CN$ = 60/40(v/v). flow rate : 0.5ml/min. column temperature : 25°C. detection : UV 254nm.



chiral separation of propranolol is seen in Figure 6. The retentions and the resolution were positively correlated and seem to be considerably dependent on the kind of the anion. The order of retention by increasing ability was as follows.

$$ClO_4^- > SCN^- > I^- > NO_3^- > Br^- > Cl^- > AcO^-$$

This order is in good agreement with that of chaotropicity[4]. Not only propranolol but also other primary, secondary and tertiary amines showed a similar dependence. The effects of cations on the retention and resolution are much different from those of the anions. That is, the increasing chaotropicity of the cation resulted in the decrease of the retention and the relationship between the chaotropicity of the cations and the resolution is unclear but small. (Figure 7).

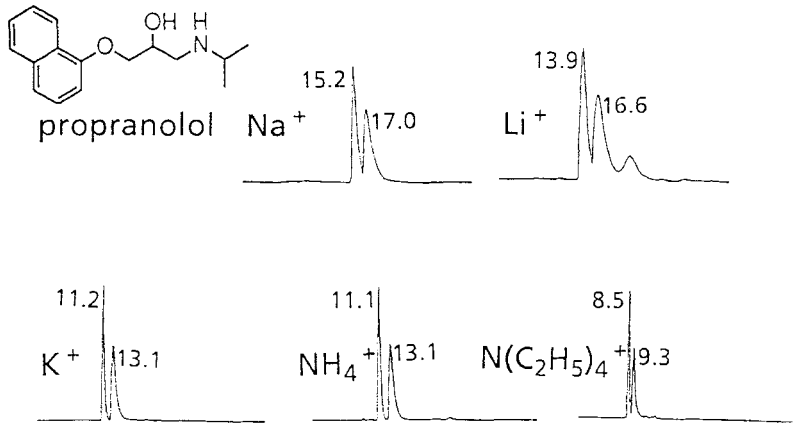


Figure 7. Effect of some chaotropic cations as moible phase additive on the separation of propranolol on a CHIRALCEL OD-R column. The retention times are given with the numbers near each peak in minutes.
mobile phase : 0.1M aq. $ClO_4^-$ salt/$CH_3CN$ = 60/40(v/v). flow rate : 0.5ml/min. column temperature : 25°C. detection : UV 254nm.



$$Na^+ > Li^+ > K^+ > NH_4^+ > N(C_2H_5)_4^+$$

From these results, we conclude that addition of a chaotropic anion is crucial to attain a good separation. These characteristics of this chromatographic system are apparently very similar to ion-pair chromatography. Though the mechanism by which a cation affects the retention is unclear, it seems reasonable that the secondary interactions among the anions and the cations in the chromatography system (i.e., van der Waals interaction, hydrogen bonding, etc.) may affect the ion pair equilibrium and eventually the retention.

There are a number of interpretations of ion-pair chromatography[5]. It seems reasonable in genaral that a chaotropic ion which is characterized by less localized electric charge, a high polarizability, and a low degree of hydration as the result of the foregoing factors is fit for partition to an organic phase, because of

the small loss of hydration enthalpy in the transfer from an aqeous phase to an organic phase. We further investigated more effective ion-pair reagents from the following characteristics : a high chaotropicity, a high charge delocalization and acidity, or a high extraction constant with an organic phase of an ion pair. Thus, we found that soduim salts of $PF_6^-$, $BF_4^-$ and $CCl_3CO_2^-$ gave a comparable or an even better resolution than that of perchlorate (Figure 8).

Our results concerning the use of sulfonate salts are also noteworthy. We examined the effect of the addition of sodium undecane and octanesulfonates as well as sodium trifluoromethanesulfonate, which seems to have much more dispersed negative charge (Figure 8). While very hydrophobic undecanesulfonate apparently endowed the analytes with a very strong retention even with low concentrations of the ion-pair reagent, the resolution was very poor because of a low separation factor and a considerable tailing. On the other hand, sodium trifluoromethanesulfonate gave a much better resolution, although it required a much greaterl concentration than that for undecanesulfonate to give a comparable retention. This fact suggests that the use of a long chain alkanesulfonate salt is accompanied by a considerable nonstereoselective adsorption. A trifluoromethanesulfonate salt was much more satisfactory than the alkanesulfonates, but still very often poorer than a perchlorate, hexafluorophosphate, tetrafluoroborate, or trichloroacetate salt when compairing the chromatographic resolution. Some other examples of the chiral separation of basic analytes are given in Figure 9.



Chiral separation of onium ion compounds.

Here, we would like to mention the chiral separation of a quarternary ammonium salt. The chiral separation of N-methylated

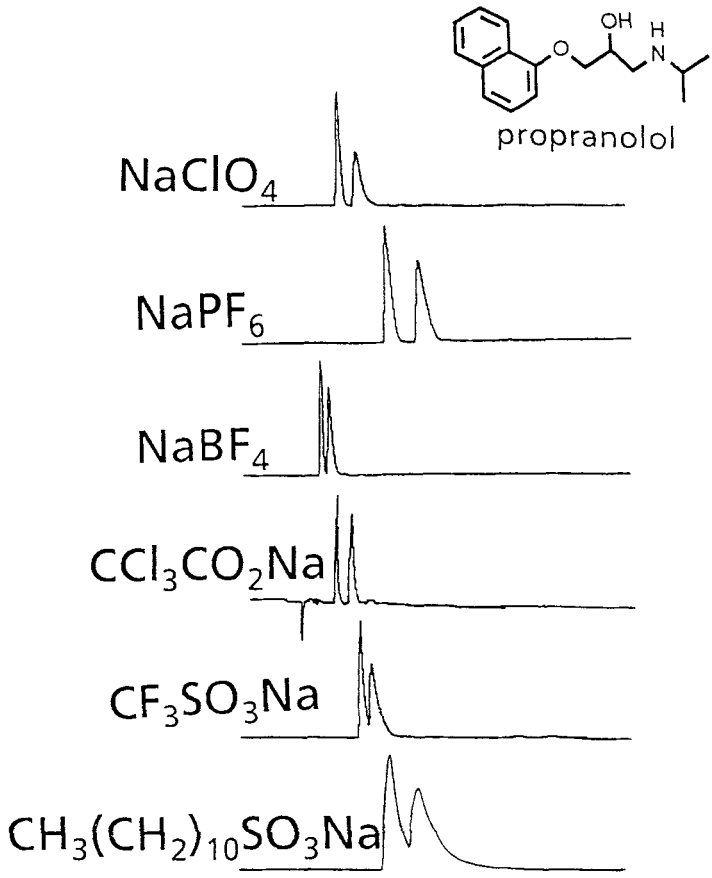


Figure 8. Effect of other mobile phase additives on the separation of propranolol on a CHIRALCEL OD-R column.
mobile phase : 0.1M ($NaClO_4$, $NaPF_6$, $NaBF_4$, $CCl_3CO_2Na$, and $CF_3SO_3Na$) or 0.02M ($CH_3(CH_2)_{10}SO_3Na$) aq. salt/$CH_3CN$ = 60/40(v/v). flow rate : 0.5ml/min. column temperature : 25°C. detection : UV 254nm.

verapamil with a reversed-phase mobile phase containing sodium perchlorate is shown in Figure 10. Ion-pair chromatography could be successfully applied to this group of compounds as well as to primary, secondary and tertiary amines. We recently found [6] that addition of trifluoroacetic acid-diethylamine salt into a normal

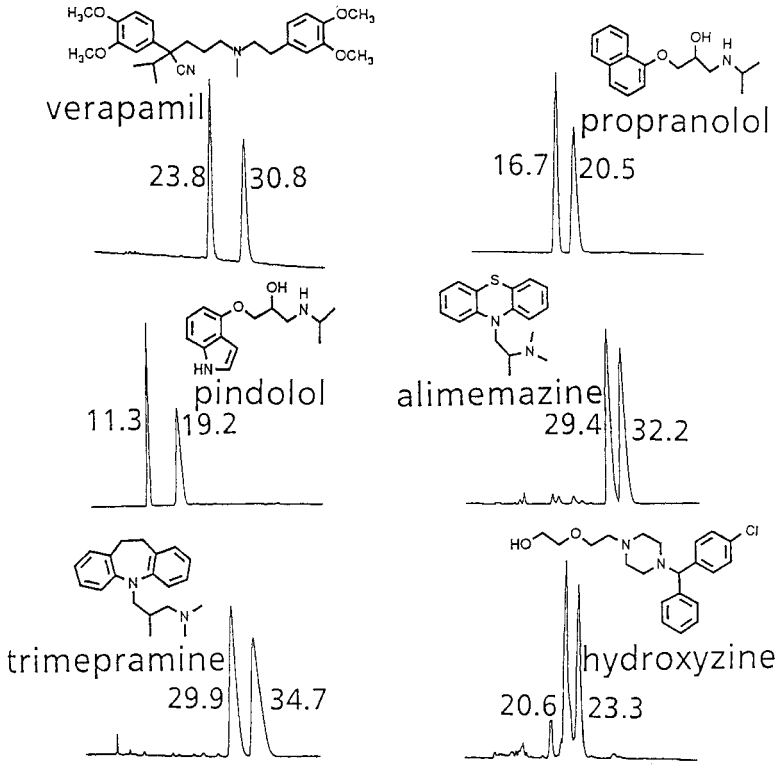


Figure 9. Chromatograms of basic compounds : verapamil, propranolol, pindolol, alimemazine, trimepramine, and hyroxyzine on a CHIRALCEL OD-R column. The retention times are given with the numbers near each peak in minutes.
mobile phase : 0.1M aq. $NaPF_6/CH_3CN$ = 60/40(v/v). flow rate : 0.5ml/min. column temperature : 25°C. detection : UV 254nm.

mobile phase resulted in a good chiral separation of tertiary sulfonic and ammonium compounds. Thus, chiral separation of this class of ionic compounds on a cellulosic phase can be attained now under both normal phase and reversed-phase conditions.

Optimization of chromatographic conditions.

In this part of the paper, we would like to mention some other factors affecting the separation as practical information to control

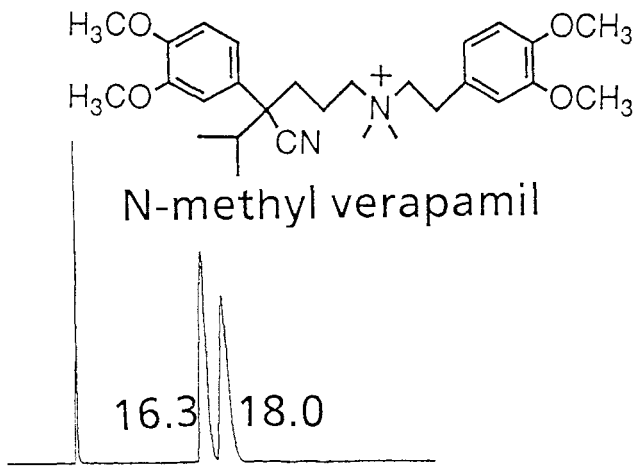


Figure 10. Chromatogram of N-methylated verapamil on a CHIRALCEL OD-R columnCHIRALCEL OD-R. The retention times are given with the numbers near each peak in minutes.
mobile phase : 0.5M aq. $NaClO_4/CH_3CN = 60/40(v/v)$. flow rate : 0.5ml/min. column temperature : 25°C. detection : UV 254nm.



the chromatographic conditions. Needless to say, chromatographic conditions should be chosen by considering the requirements for the analysis, i.e., precision, time, instrument etc.. i) Concentration of ion-pair reagent : The dependence of the chromatograms of propranolol and trimepramine on sodium perchlorate concentration is shown in Figure 11. The retentions and the resolutions increased with the increase of the salt concentration and did not saturate even at a 4M concentration. ii) Water / organic solvent ratio : As usual with reversed-phase chromatography, the retention increased with an increase of the water / organic solvent ratio. iii) Temperature : The effect of temperature on the chiral separation of propranolol and trimepramine can be seen in Figure 12. A lower temperature favored the separation. iv) Choice of organic solvent : A comparison of acetonitrile and methanol was

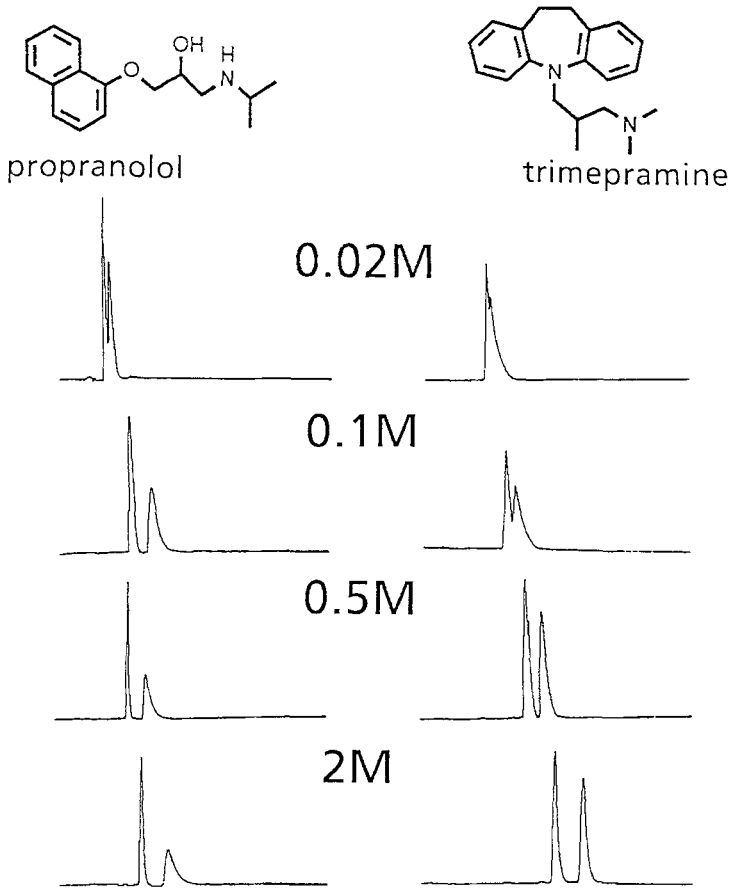


Figure 11. Effect of concentration of $NaClO_4$ on the separation of propranolol and trimepramine on a CHIRALCEL OD-R column.
mobile phase : aq. $NaClO_4/CH_3CN$ = 60/40(v/v). flow rate : 0.5ml/min. column temperature : 25°C. detection : UV 254nm.

made (Figure 13). In the cases of propranolol and verapamil, similar retentions were obtained with 40% aq. acetonitrile and with 70% aq. methanol. However, with the methanolic mobile phase, the elution peaks were considerably broader than those obtained with the acetonitrile mobile phase and eventually the resolutions were

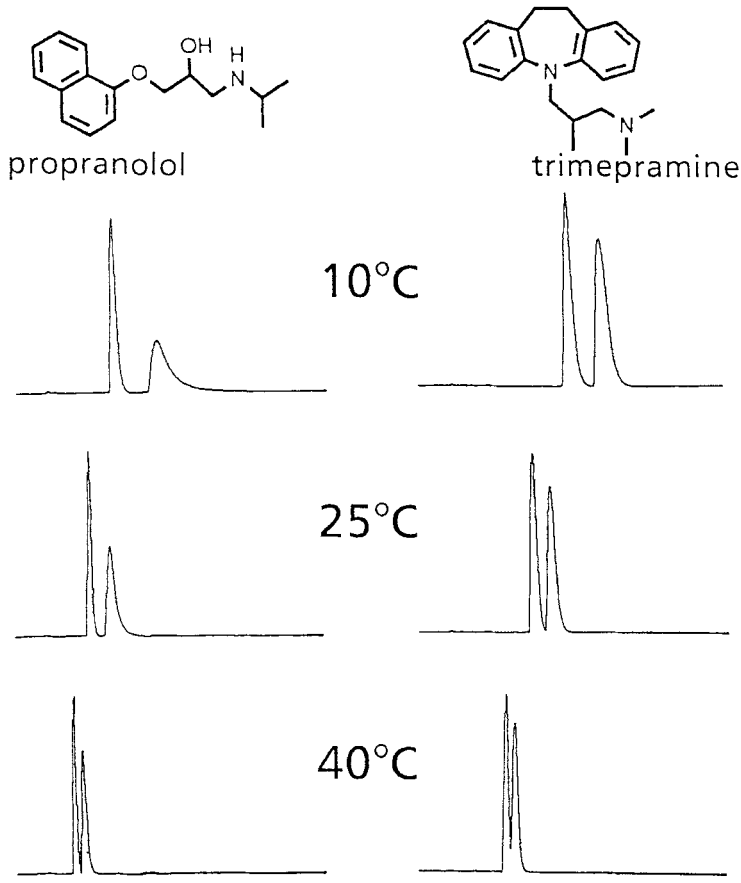


Figure 12. Effect of column temperature on the separation of propranolol and trimepramine on a CHIRALCEL OD-R column.
mobile phase : 0.1M aq. $NaClO_4/CH_3CN = 60/40(v/v)$. flow rate : 0.5ml/min. detection : UV 254nm.

poorer. Sevral analytes were employed in each study and, so far, no exceptions have been found.

Column stability.

The stability of CHIRALCEL OD-R columns (25cm × 0.46cm$\phi$) was examined for 100hrs under a flow rate of 1.0ml/min (The total

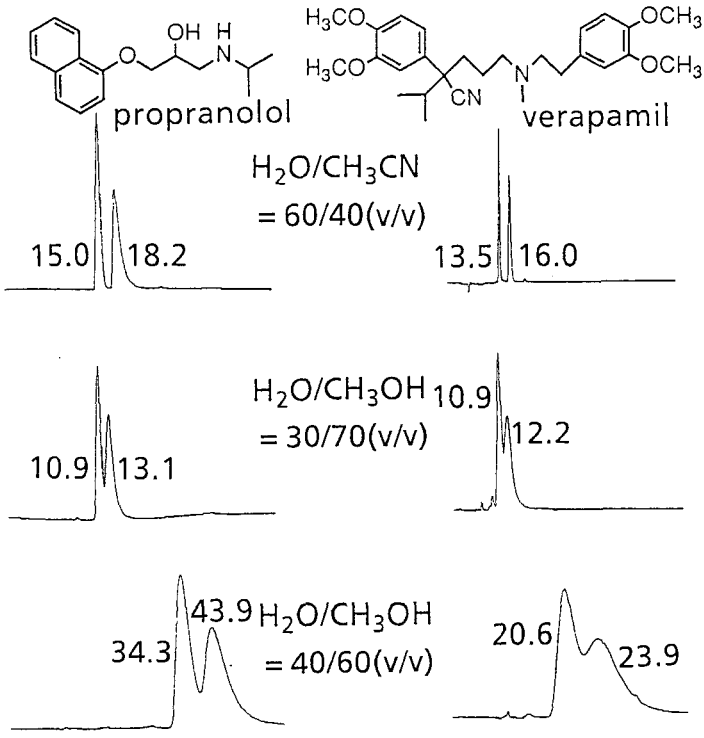


Figure 13. Comparison of acetonitrile and methanol as the organic constituent of the mobile phase on the separation of propranolol and verapamil on a CHIRALCEL OD-R column. The retention times are given with the numbers near each peak in minutes.
mobile phase : 0.5M aq. $NaClO_4/CH_3CN$ or $CH_3OH$. flow rate : 0.5ml/min. column temperature : 25°C. detection : UV 254nm.

volume was ca. 6L.) and at 40°C with the following mobile phases : (1) acetonitrile (2) methanol (3) water (4) aq. $HClO_4$-$NaClO_4$ (0.05M, pH 2) (5) aq. $HClO_4$-$NaClO_4$ (0.05M, pH 2) / $CH_3CN$ = 60/40(v/v) (6) aq. HOAc-NaOAc (0.05M, pH 7) (7) aq. $KH_2PO_4$-$Na_2HPO_4$ (0.05M, pH 7) (8) aq. $KH_2PO_4$-$Na_2HPO_4$ (0.05M, pH 8). Though no deterioration of column performance was observed with mobile phases (1)~(5), distortion of the elution peaks was found with mobile phase (6)~(8). A buffered mobile phases at pH 7 appears to damage the

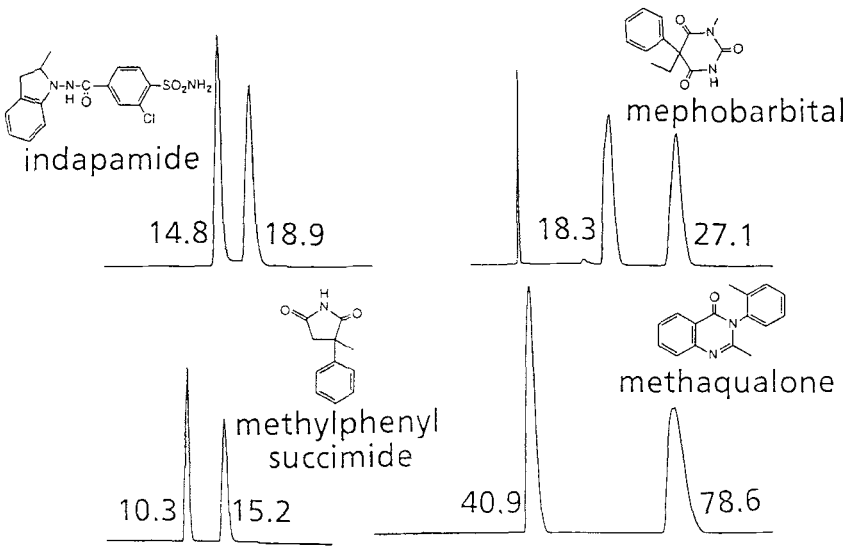


Figure 14. Chromatograms of neutral compounds : indapamide, mephobarbital, methylphenylsuccimide, and methaqualone on a CHIRALCEL OJ-like column. The retention times are given with the numbers near each peak in minutes. mobile phase : $H_2O/CH_3CN = 60/40(v/v)$. flow rate : 0.5ml/ min. column temperature : 40°C. detection : UV 254nm.



column within 100hrs but an unbuffered one of the same pH does not ; further, the column is highly durable to an acidic mobile phase (pH 2).

Other columns.

The practical usefulness of a polysaccharide-based column was confirmed not only with a cellulose tris(3,5-dimethylphenylcarbamate)-based column (CHIRALCEL OD-R) but also with other polysaccharide-based columns. For example, several neutral pharmaceuticals were resolved on a cellulose tris( 4-methylbenzoate)-based column (CHIRALCEL OJ-like) with aq.

acetonitrile as the mobile phase (Figure 14). The column performance was stable with 0.05M aq. $NaClO_4$-$HClO_4/CH_3CN = 80/20$(v/v) (flow rate : 1.0 ml/min.) at 40°C for at least 100hrs. The detailed study of columns other than CHIRALCEL OD-R are in progress, but they are not yet commercially available.

## Conclusion.

A CHIRALCEL OD-R column was proven to be practical for purposes useful under reversed-phase conditions. To attain a satisfactory chiral separation, the chromatographic conditions must be carefully chosen, to include an ion-pair chromatography condition for the separation of an amine and ionization control for that of a carboxylic acid. It should be theoretically possible to apply ion-pair chromatography to an acidic analyte and apply ionization control to a basic one. Though the studies have not yet been conducted, such conditions should be of limited practical usefulness because of the column deterioration with an aqueous mobile phase of a high pH (pH > 7.0).

We think the effect of ionic mobile phase additives in normal phase chromatography is worth mentioning. Okamoto et al. reported that a mobile phase acidified with trifluoroacetic acid is suitable for the separation of an acidic analyte[7] and one made basic with diethylamine to that of a basic analyte[1-d], and their practical usefulness is well confirmed. However, R. Gaskell[8] reported that an acidified mobile phase gave a good separation of a polar basic compound and we succeeded in resolving a tertiary sulfonium compound with a mobile phase containing trifluoroacetate salt. This suggests that the concept of ion-pair chromatography may be applicable not only under a reversed-phase condition but also under a normal phase condition.

## ACKNOWLEDGEMENTS

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