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# Conalbumin-conjugated silica gel, a new chiral stationary phase for high-performance liquid chromatography

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ABSTRACT

A new chiral stationary phase using conalbumin (from chicken egg white) was developed for high-performance liquid chromatography. Chiral resolution of racemic azelastine, an antiallergic drug, was achieved on a conalbumin-conjugated silica gel column. The effects of the pH, the concentration of organic solvents and salts in the mobile phase, and the temperature on the capacity factor and resolution of racemic azelastine were examined. This column shows good stability and can separate optical isomers with an aqueous mobile phase. It should be very useful in studies on pharmacokinetics and in clinical chemistry.

## INTRODUCTION

Chiral discrimination has been a problem in the development and use of pharmaceutical drugs, because drug enantiomers can have different pharmacokinetic properties and cause different physiological responses. For this reason, many studies on optical resolution by high-performance liquid chromatography (HPLC) have been conducted, and the direct resolution of racemic compounds has been achieved by use of chiral stationary phases. However, many of them are used under normal-phase conditions, and laborious pretreatments are required to eliminate water in samples. At present cyclodextrin-conjugated columns [1] and protein-conjugated columns, which can be used in reversed-phase mode, are commercially available. The usefulness of protein-conjugated columns in HPLC has been demonstrated by Allenmark *et al.* [2] and Hermansson [3]. Allenmark *et al.* [2] have successfully resolved acidic compounds by using a bovine serum albumin-conjugated column, and Hermansson [3] resolved racemic amines with an $\alpha_1$-acid glycoprotein-conjugated column. However, these columns do not have sufficient durability. Miwa *et al.* [4] have developed a highly effective column for chiral recognition by using ovomucoid, an acid glycoprotein found in chicken egg white. An ovomucoid col-

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umn can achieve the chiral resolution of acidic and basic compounds [5,6], is quite resistant to variations in pH, to heat and to organic solvents [4,7], and has been proved to have higher stability and chiral resolution than the $\alpha_1$-acid glycoprotein-conjugated column described by Kirkland *et al.* [8]. Oda *et al.* [9] have recently performed on-line simultaneous determination and resolution of enantiomers of verapamil and its metabolites in plasma by employing an ovomucoid column with a column-switching technique.

In this study we used azelastine (AZE), which is a phthalazinone derivative, as a model compound. AZE is an antiallergic drug with a wide spectrum of pharmacological activities. It inhibits the action of many chemical mediators such as leukotriene [10] and histamine [11]. AZE has an asymmetric carbon (Fig. 1), and Kajima *et al.* [12] have achieved the separation of enantiomeric AZE by ion-pair chromatography under normal-phase conditions. However, optical resolution of this drug has not been achieved by HPLC with an aqueous mobile phase, which would be required for studies of the pharmacokinetics of enantiomeric AZE by HPLC. In this paper, we describe a new stationary phase for chiral resolution, conalbumin-conjugated silica gel, which allows the separation of AZE enantiomers by means of reversed-phase HPLC.

## EXPERIMENTAL

### *Apparatus*

A Shimadzu LC-9A pump (Shimadzu, Kyoto, Japan) equipped with an SPD-6A variable-wavelength UV monitor was used. A stainless-steel column of 150 mm × 4.6 mm I.D. was packed with conalbumin-conjugated silica gel. The sample was injected with a Model 7125 (Rheodyne) injector. The pH was measured with a TDA HM-60S pH meter (TOA Electronics, Tokyo, Japan).

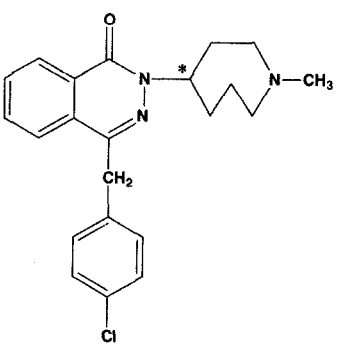


Fig. 1. Structure of *d,l*-azelastine. The chiral centre is indicated by an asterisk.

### *Preparation of conalbumin column*

Conalbumin-conjugated silica gel was prepared as follows: Unisil Q $NH_2$ (2 g) and N,N-dissucinimidyl carbonate (3 g) were made to react for 6 h in acetonitrile (50 ml) at room temperature using a magnetic stirrer. The activated silica gel was washed with acetonitrile and then with the coupling buffer (50 m*M* potassium phosphate buffer, pH 7.5). Conalbumin (2 g) was dissolved in 50 ml of coupling buffer and then the activated silica gel was added. The mixture was stirred for 6 h at room temperature with a magnetic stirrer. After reaction, the conalbumin-conjugated silica gel was collected by filtration and washed with water and 2-propanol–water (1:2), then packed into a stainless-steel column (150 mm × 4.6 mm I.D.) by a conventional high-pressure slurry-packing procedure.

### *Reagents and materials*

Racemic AZE [(+)-4-(4-chlorobenzyl)-2-(hexahydro-1-methyl-1H-azepin-4-yl)-1(2H)-phthalazinone] and optically active AZE were prepared in our laboratories (Fig. 1). Conalbumin was purified from chicken egg white. N,N-Disuccinimidyl carbonate was purchased from Wako Pure Chemical Industries (Osaka, Japan). Unisil Q $NH_2$ was purchased from Macherey-Nagel (Düren, Germany). Organic solvents and water were of HPLC grade.

### *Sample*

A known amount of racemic AZE was dissolved in methanol and the solution was diluted with water to a concentration of 20 ng/μl.

## RESULTS AND DISCUSSION

Conalbumin is an egg-white protein that is also known as ovotransferrin. This protein binds iron, copper, manganese and zinc [13–15] at pH 6 or above, and has an action that blocks the growth of bacteria. Its molecular weight is about 70 000–78 000, and its p*I* value is 6.1–6.6. In this study we

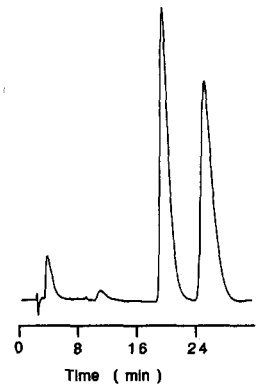


Fig. 2. Separation of *d,l*-azelastine on a conalbumin column. Chromatographic conditions: mobile phase, 50 m*M* potassium phosphate buffer (pH 5.0) containing 8% ethanol; detection, UV 230 nm; flow-rate, 1.0 ml/min; column temperature, room temperature; sample amount, 200 ng.

prepared conalbumin-conjugated silica gel as an HPLC stationary phase, and used the basic compound AZE as a model compound for chiral separation. The optimum conditions for chiral resolution were found to be 50 m*M* potassium phosphate buffer (pH 5.0) containing 8% ethanol, as shown in Fig. 2.

In this column, the pH of the mobile phase greatly affected the capacity factor ($k'$ values) and chiral separation, as shown in Table I. The $k'$ values of racemic AZE increased with an increase in the pH. The p$K$ value of AZE is around 8.5, as determined by neutralization analysis, so its hydrophobicity should not change much in the pH range 4–7. As noted above, the p$I$ value of conalbumin is 6.1–6.6, so the hydrophobicity of this solid phase is maximum in this pH range, and the retention of AZE is strongest at around pH 6.5 in the pH range 3.0–6.5. The reason for this steems to be related to the dissociation of the carboxylic acid moieties of amino acids and the sialic acid moieties of the glycoprotein (conalbumin). On the other hand, the separation factor ($\alpha$) and resolution ($R_s$) of AZE were almost constant at pH 4.5–6.5. It was very interesting that the $\alpha$-value and $R_s$ value were not greatly changed in the pH range 4.5–6.5, although the retention was decreased with a decrease in pH in this range. This seems to show that the interaction between AZE and the chiral recognition site was not changed in spite of variation in the hydrophobicity of the total protein molecule. That is to say, the chiral recognition site of this column for AZE may be dissociated if it has an acidic function or not dissociated if it has a basic function, and its p$K$ value may be around 4.5. So the enantioseparation for AZE may constant in this pH range in spite of the decrease in the retention with decreasing pH.

The performance of this column was also affected by the concentration of salts in the mobile phase (pH 5.0). As shown in Table II, the $k'$ values of AZE increased with increasing salt concentration, though the $\alpha$-value was not much changed. In general, the hydrophobicity of drugs is little affected by the salt concentration of phosphate buffer in

TABLE I

EFFECT OF THE pH OF THE MOBILE PHASE ON ENANTIOSELECTIVITY FOR *d,l*-AZELASTINE ON THE CONALBUMIN COLUMN

$k'_1$ = capacity factor of *d*-azelastine; $k'_2$ = capacity factor of *l*-azelastine; $n$ = plate number of *d*-azelastine. Chromatographic conditions: the pH of the mobile phase was as shown in the table, and other conditions were the same as in Fig. 2.

| pH | $k'_1$ | $k'_2$ | $\alpha$ | $R_s$ | $n$ |
|---|---|---|---|---|---|
| 3.0 | 0.70 | 0.70 | 1.00 | 0.00 | 908 |
| 3.5 | 1.41 | 1.64 | 1.17 | 0.98 | 1313 |
| 4.5 | 5.15 | 6.50 | 1.26 | 1.84 | 1346 |
| 5.5 | 10.32 | 13.60 | 1.32 | 1.99 | 1138 |
| 6.0 | 16.58 | 21.30 | 1.29 | 1.92 | 968 |
| 6.5 | 26.55 | 34.59 | 1.30 | 1.86 | 617 |

TABLE II

EFFECT OF THE CONCENTRATION OF SALTS IN THE MOBILE PHASE ON ENANTIOSELECTIVITY FOR *d,l*-AZELASTINE ON THE CONALBUMIN COLUMN

$k'_1$ = capacity factor of *d*-azelastine; $k'_2$ = capacity factor of *l*-azelastine; $n$ = plate number of *d*-azelastine. Chromatographic conditions: the concentration of salts in the mobile phase was as shown in the table, and other conditions were the same as in Fig. 2.

| Concentration (m*M*) | $k'_1$ | $k'_2$ | $\alpha$ | $R_s$ | $n$ |
|---|---|---|---|---|---|
| 5 | 2.39 | 2.75 | 1.15 | 0.95 | 823 |
| 10 | 3.06 | 3.57 | 1.17 | 0.99 | 852 |
| 20 | 4.31 | 5.12 | 1.19 | 1.17 | 802 |
| 50 | 6.14 | 7.11 | 1.16 | 1.06 | 921 |
| 100 | 7.68 | 8.83 | 1.15 | 1.09 | 1061 |
| 250 | 8.82 | 9.93 | 1.13 | 1.03 | 1345 |
| 500 | 11.45 | 12.76 | 1.12 | 0.82 | 1027 |

HPLC. So the increase in $k'$ values may be related to the increase in hydrophobicity of the solid phase by the salting out of conalbumin, although the chiral recognition ability was not greatly changed.

This column was also very much affected by the concentration of organic solvents in the mobile phase; the separation mode of this column was reversed phase, as shown in Fig. 3. The hydrophobic retention of AZE on the conalbumin column was very weak compared with that on an octadecylsilane (ODS) column, and the mechanism of the retention in this column seems to involve various interactions, such as hydrophobic and ionic interactions, between conalbumin and AZE. Table III shows the effect of mobile phase modifiers on $k'$ values and enantioselectivity. When the $k'$ values of methanol, ethanol, 1-propanol and 1-butanol were compared, they clearly increased with decreasing hydrophobicity of the organic solvents, and this result agrees well with that in Fig. 3. In the cases of methanol and ethanol, lower hydrophobicity gave better $R_s$ values than were obtained with 1-propanol or 1-butanol. But in the case of branched-chain organic solvents such as 2-propanol or *tert.*-butanol, the above rule did not necessarily hold. As shown in Table III, *tert.*-butanol, which has a larger molecular weight, was more strongly retained than 2-propanol, and *tert.*-butanol likewise had a better $R_s$ value than 2-propanol. Acetonitrile, lacking a hydroxyl group, gave poor resolution of racemic AZE, its α-value, 1.21, being almost the same as that of *tert.*-butanol, 1.23. But its $R_s$ value was only 0.72. It is clear that the resolution of the conalbumin column depends strongly on the kind of organic solvents used. Therefore we consider that organic solvents must be carefully selected whenever a pro-

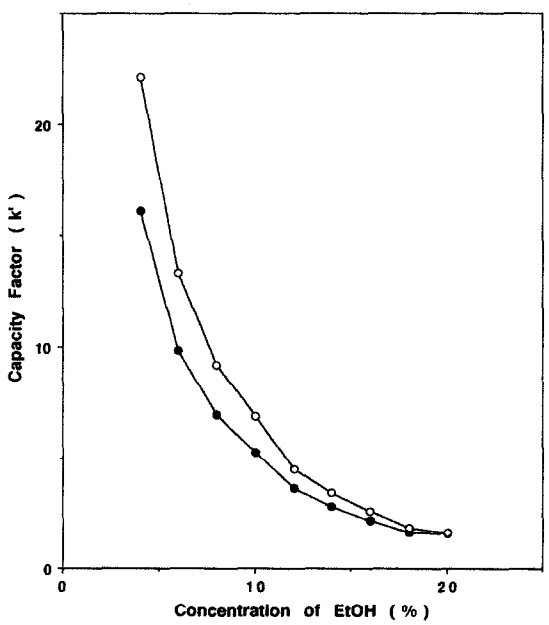


Fig. 3. Effect of concentration of ethanol (EtOH) in the mobile phase on capacity factor of *d,l*-azelastine on the conalbumin column. ● = *d*-azelastine; ○ = *l*-azelastine. Chromatographic conditions: concentration of ethanol in the mobile phase was as shown in the figure and other conditions were the same as in Fig. 2.

TABLE III

EFFECT OF THE MOBILE PHASE MODIFIERS ON ENANTIOSELECTIVITY FOR *d,l*-AZELASTINE ON THE CONALBUMIN COLUMN

$k'_1$ = capacity factor of *d*-azelastine; $k'_2$ = capacity factor of *l*-azelastine; $n$ = plate number of *d*-azelastine. Chromatographic conditions: the concentration of organic solvents was 8%, and other conditions were the same as in Fig. 2.

| Solvents | $k'_1$ | $k'_2$ | $\alpha$ | $R_s$ | $n$ |
|---|---|---|---|---|---|
| Methanol | 14.21 | 21.17 | 1.49 | 1.61 | 270 |
| Ethanol | 6.92 | 9.17 | 1.33 | 1.78 | 1238 |
| 1-Propanol | 3.04 | 3.24 | 1.07 | 0.30 | 342 |
| 2-Propanol | 5.39 | 6.74 | 1.25 | 1.05 | 507 |
| 1-Butanol | 3.01 | 3.01 | 1.00 | 0.00 | 1160 |
| *tert.*-Butanol | 6.67 | 8.18 | 1.23 | 2.01 | 1082 |
| Acetonitrile | 3.59 | 4.34 | 1.21 | 0.72 | 497 |

TABLE IV

EFFECT OF COLUMN TEMPERATURE ON ENANTIOSELECTIVITY FOR *d,l*-AZELASTINE ON THE CONALBUMIN COLUMN

$k'_1$ = capacity factor of *d*-azelastine; $k'_2$ = capacity factor of *l*-azelastine. Chromatographic conditions: the column temperature was given in the table, and other conditions were the same as in Fig. 2.

| Temperature (°C) | $k'_1$ | $k'_2$ | $\alpha$ | $R_s$ |
|---|---|---|---|---|
| 4 | 23.87 | 28.86 | 1.21 | 1.06 |
| 10 | 16.79 | 20.43 | 1.22 | 1.08 |
| 20 | 9.26 | 11.11 | 1.20 | 1.12 |
| 25 | 7.31 | 8.56 | 1.17 | 1.08 |
| 30 | 6.08 | 6.95 | 1.14 | 0.94 |
| 37 | 4.87 | 5.40 | 1.11 | 0.72 |

tein-conjugated column such as a conalbumin column is used.

In the conalbumin column, the retention of racemic AZE decreased with an increase in the column temperature (Table IV). This may reflect the partition of molecules into the mobile phase, leading to an increase in partition ratio into the aqueous phase at high temperature. However, the α-value was not much changed. Considering the influence of the column temperature on the stereoselectivity and on the chromatographic efficiency, the optimum temperature was around 20°C in the analysis of AZE.

Conalbumin is labile to heat and acid, unlike chicken egg-white ovomucoid, which is relatively resistant to variation in pH, to heat and to organic solvents. However, conalbumin-conjugated silica gel was stable as a stationary phase for HPLC. Because the conalbumin-coupled silica gel column was injected with samples 400–500 times over 3 months, and although the $R_s$ values of racemic AZE decreased from 1.7 to 1.1, the chiral recognition ability of this column was well retained. This seems to be related to the interesting property of conalbumin that it is stabilized to heat when combined with iron, copper, manganese and zinc [13–15]. That is to say, the conformation of this protein may be fixed by complexation with these metals. Thus, we considered that fixing the conformation of this protein by conjugating it to silica gel would improve its stability, and we expect that this protein can be used as a reversed phase for chiral resolution by HPLC. Even better durability may be attainable by using another method of fixation of conalbumin.

In a protein-conjugated column, the loading limit is generally much lower than that in an ODS column, because the conjugated protein only covers the external region of silica gel, and only limited regions of the protein are effective for chiral recognition. As expected, the conalbumin column showed a low loading limit. The $R_s$ value when 200 ng were injected was about 1.1, and the $R_s$ value when 1 μg was injected about 0.9 (only 20% less). In addition, all retentions were almost the same. However, when 10 μg of racemic AZE were injected, the isomers were not resolved in this column.

The chiral resolution of racemic AZE has not previously been achieved directly by using reversed-phase HPLC. The implication is that chiral resolution ability varies greatly according to the nature of the protein conjugated to silica gel. If the relation between ligand structure and drug structure can be better understood, it should be possible to prepare columns with highly efficient chiral recognition ability for specific purposes.

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