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Application of an ovomucoid-conjugated polymer column for the enantiospecific determination of chlorprenaline concentrations in plasma

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ABSTRACT

An ovomucoid-conjugated polymer column was prepared for the liquid chromatographic resolution of racemic compounds. The column showed strong retention of acidic solutes, a characteristic attributed to the structure of the stationary phase support gel. Although the efficiency of the column was lower than that of an ovomucoid-conjugated silica gel column, enantiospecific chlorprenaline determination in plasma was achieved with solute amounts from 1.0 ng to 0.1 μg.

INTRODUCTION

The development of chiral resolution columns has tended to be directed mainly toward the reversed-phase type. The advantages of reversed-phase columns are easy application to assays of biomedical samples and substantial savings in operating costs, including those of chromatographic condition set-up. It should also be noted that high-sensitivity analyses using short-wavelength UV determinations are possible by reversed-phase column liquid chromatography. Protein-bonded columns [1] and cyclodextrin columns [2], which are the two most useful reversed-phase chiral columns, and cellulose triacetate columns [3] have been used for the determination of drugs in plasma. The enantiospecific pharmacokinetic data required by the Food and Drug Administration for new drug applications [4] emphasize the importance of these columns.

All the protein-bonded chiral columns now on the market use silica gel as a

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stationary phase support. The number of plates (N) reported for the protein-bonded phases is less than 2000. This figure is much lower than that for ordinary reversed-phase silica gel columns, which are said to be useful for attaining high column efficiency; however, it is not essential to use silica gel for chiral stationary phase supports.

Synthetic polymers that have almost the same N as silica gel are now commercially available. The stability of these polymers over a wide pH range, when they are used as the stationary phase for protein binding, makes possible the chromatography of basic compounds around their pK_a.

We have applied the acidic chicken egg-white protein, ovomucoid, which is stable to heat, pH variation and organic solvents, to a chiral stationary phase [5], and the resulting column is now on the market. This paper describes the preparation of an ovomucoid-conjugated polymer column and its retention characteristics. We also report the use of the column for the enantiospecific determination of chlorprenaline (which, because of its β-adrenergic effect, is used as a bronchodilating agent) in biological fluids.

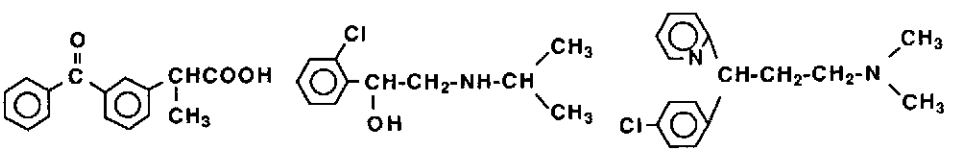
EXPERIMENTAL

Apparatus

A Shimadzu LC-6A pump equipped with an SPD-6A variable-wavelength UV monitor and an SCL-6A automatic sample injector was used. A Shimadzu C-R4A Chromatopac was employed as a data processor, and a Jasco Multi-330 multi-channel detector was also used. The zeta potentials of ovomucoid-conjugated silica gel and ovomucoid-conjugated polymer gel were measured with a Penkem Laser Zee Model 501, from a suspension of 50 mg of each gel in 100 ml of water.

Chemicals

The amino residue-conjugated polymer, Asahipak Gel NH2P-50, was purchased from Asahi Chemical Industries (Kawasaki, Japan). Chlorprenaline from Eisai (Tokyo, Japan), chlorpheniramine maleate from Kowa (Nagoya, Japan), ketoprofen from Nihon Bulk Yakuhin (Osaka, Japan) and N,N-disuccinimidyl carbonate from Wako (Kyoto, Japan) were used. The structures of chlorpheniramine, chlorprenaline and ketoprofen are shown in Fig. 1. (+)-Chlorpheniramine



Ketoprofen **Chlorprenaline** **Chlorpheniramine**

Fig. 1. Structures of compounds resolved by HPLC on an ovomucoid-conjugated polymer column.

and (−)-chlorprenaline were purified by differential crystallization. All other chemicals were of analytical-reagent grade or higher quality. The ovomucoid-conjugated silica gel column used, Ultron ES-OVM (15 cm × 0.46 cm I.D., 5 μm particle size) was purchased from Shinwa Chemical Industries (Kyoto, Japan).

Preparation of ovomucoid-conjugated polymer column

The principle of the synthesis of the ovomucoid-conjugated polymer was the same as that of ovomucoid-conjugated silica gel [5]. Briefly, Asahipak Gel NH2P-50 (4 g) and N,N-disuccinimidyl carbonate (8 g) reacted overnight in a coupling buffer (0.1 *M* $NaHCO_3$, pH 6.8) at room temperature with stirring. After the activated polymer had been washed with water and then with the coupling buffer, 30 ml of ovomucoid solution (2 g in 30 ml of coupling buffer) were added and the solution was stirred for 2 h at room temperature. The amount of ovomucoid-conjugated (110 mg/g of gel) was determined by substracting the amount recovered by washing from the amount used for conjugation.
The packing of the ovomucoid-conjugated polymer was executed according to the instruction for the Asahipak gel: 2 g of the gel were suspended in 50 ml of 20 m*M* potassium phosphate buffer (pH 6.2), and the suspension was slurry-packed into a steel column (15 cm × 0.46 cm I.D.).

Enantiospecific determination of chlorprenaline in plasma

Samples (1 ml) of human plasma spiked with known amounts of chlorprenaline were transferred to glass screw-cap tubes, to which 20 μl (500 ng) of an aqueous (+)-chlorpheniramine solution as the internal standard and 1 ml of distilled water were added. The mixture was extracted with 5 ml of diethyl ether for 20 min on a reciprocating shaker. After centrifugation (1000 *g*, 5 min), the aqueous layer was frozen in a dry ice–acetone bath, and the diethyl ether was decanted into a clean tube. A second extraction was performed in the same way. The combined ethereal layer was evaporated to dryness under a stream of nitrogen. The residue was reconstituted by vortex-mixing in 200 μl of the mobile phase, and a portion of 20–50 μl was injected.

RESULTS

The pH-retention characteristics and the effect of ethanol concentration on the retention of maleic acid, chlorprenaline, chlorpheniramine and ketoprofen in an ovomucoid-conjugated polymer (Ovm-Poly) column and in an ovomucoid-conjugated silica gel (Ovm-Sil) column are shown in Figs. 2–5. The retention of acidic solutes increased markedly when an Ovm-Poly column was used. Although pronounced retention of acidic solutes by mobile phases of lower pH was observed, the mobile phase modifier (ethanol) showed little effect on the retention of maleic acid. The retention capacity of amines on Ovm-Poly was two to three times larger than that on Ovm-Sil.

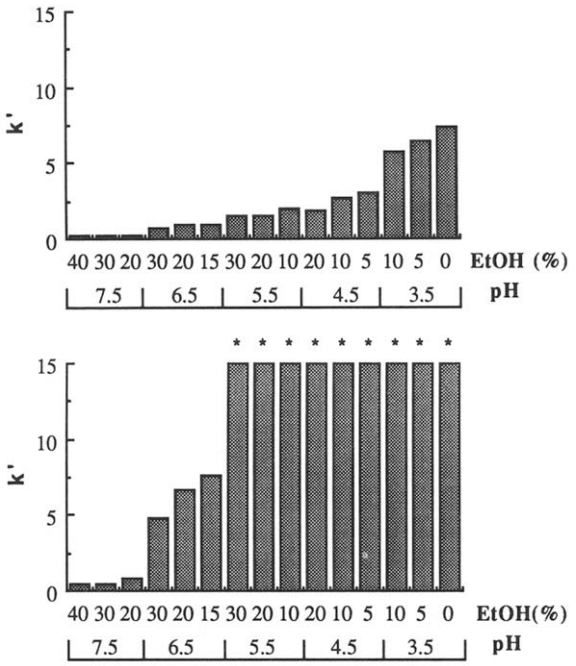


Fig. 2. Influence of pH and ethanol concentration on the retention of maleic acid: (upper) ovomucoid silica gel column; (lower) ovomucoid polymer column. Mobile phase, 20 m*M* potassium phosphate; column temperature, 30°C; flow-rate. 0.8 ml/min. Asterisks indicate values greater than 15.

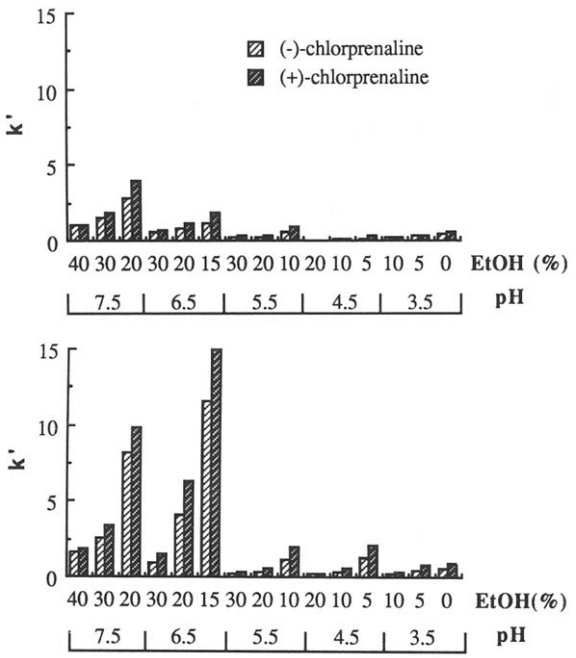


Fig. 3. Influence of pH and ethanol concentration on the retention of chlorprenaline enantiomers: (upper) ovomucoid silica gel column; (lower) ovomucoid polymer column. Chromatographic conditions as in Fig. 2.

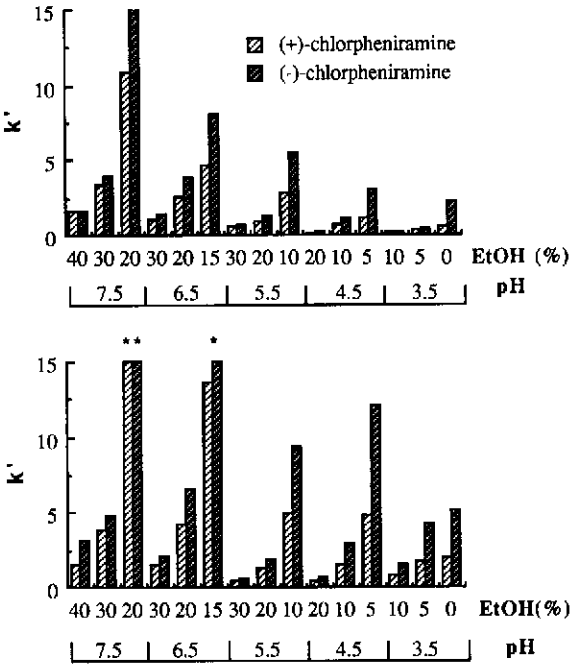


Fig. 4. Influence of pH and ethanol concentration on the retention of chlorpheniramine enantiomers: (upper) ovomucoid silica gel column; (lower) ovomucoid polymer column. Chromatographic conditions as in Fig. 2. Asterisks indicate values greater than 15.

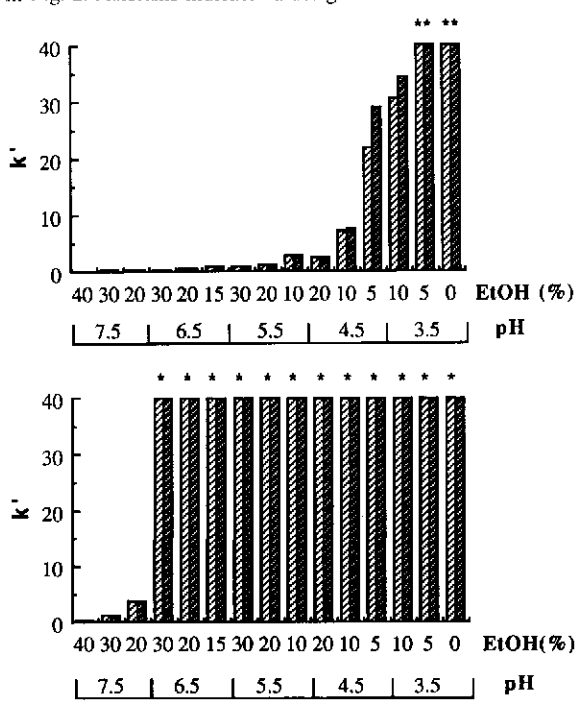


Fig. 5. Influence of pH and ethanol concentration on the retention of ketoprofen enantiomers: (upper) ovomucoid silica gel column; (lower) ovomucoid polymer column. Chromatographic conditions as in Fig. 2. Asterisks indicate values greater than 40.

The zeta potential of Ovm-Poly was −24.51 mV, and that of Ovm-Sil was −3.53 mV.

Table I shows the dependency of the capacity factor (k'), enantioselectivity (α), resolution (R_s) and column efficiency (N) on the loading amount of chlorprenaline on an Ovm-Poly column. The α and k' values were almost constant despite the variation of the loading amount from 0.004 to 1 nmol; the R_s and N values decreased as the solute concentration and the injection volume increased. The calibration curve was linear over the concentration range shown in the table;

TABLE I

DEPENDENCE OF THE CAPACITY FACTOR (k'), ENANTIOSELECTIVITY (α), RESOLUTION (R_s) AND THEORETICAL PLATE NUMBER (N) ON THE LOADED AMOUNT OF CHLORPRENALINE ENANTIOMERS ON AN OVOMUCOID-CONJUGATED POLYMER COLUMN

Solute concentration (mM)	Injection volume (μl)	k_1'	α	R_s	N
0.0004	5	–[a]	–	–	–
	10	1.15	1.64	1.08	1880
	20	1.13	1.67	0.98	1280
	50	1.15	1.60	0.94	1430
	100	1.20	1.59	0.72	1100
0.002	5	1.17	1.58	1.14	2210
	10	1.16	1.64	1.09	1880
	20	1.12	1.68	0.96	1280
	50	1.18	1.60	0.84	1020
	100	1.20	1.59	0.70	700
0.004	5	1.14	1.61	1.13	2210
	10	1.16	1.61	0.96	1670
	20	1.17	1.61	0.90	1280
	50	1.17	1.61	0.75	1080
	100	1.19	1.60	0.72	750
0.02	5	1.16	1.61	0.91	1800
	10	1.16	1.61	0.87	1650
	20	1.17	1.61	0.92	1280
	50	1.17	1.60	0.79	1010
	100	1.19	1.60	0.65	660
0.04	5	1.16	1.61	0.90	1670
	10	1.16	1.60	0.77	1120
	20	1.16	1.61	0.81	1000
	50	1.17	1.59	0.71	960
	100	1.17	1.58	0.50	680
0.2	5	1.15	1.61	0.80	1000
	10	1.14	1.61	0.74	880
	20	1.13	1.60	0.65	690
	50	1.12	1.57	0.42	340

[a] Below detection limit.

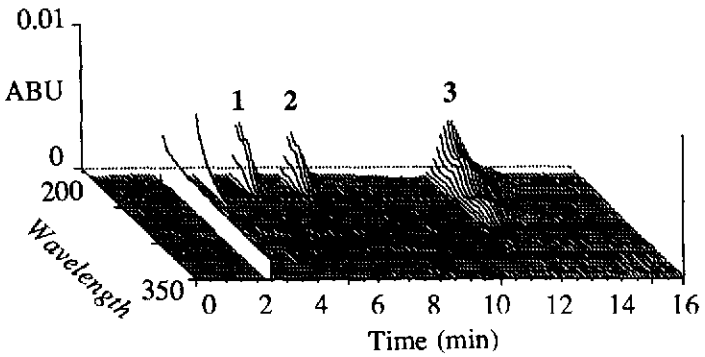


Fig. 6. Chromatogram of an extract of a 1-ml plasma sample spiked with 500 ng of racemic chlorprenaline. Peaks: 1 = (−)-chlorprenaline; 2 = (+)-chlorprenaline; 3 = internal standard. HPLC conditions: column, ovomucoid-conjugated polymer column; mobile phase, 20 m*M* potassium phosphate (pH 6.5) containing 20% ethanol; flow-rate, 0.8 ml/min; column temperature, 33°C.

correlation coefficients were between 0.996 and 0.999, when the peak area of each enantiomer was plotted against the injected amount of racemic chlorprenaline. The value of N obtained at an injection volume of 50 μl was 1.5–2.5 times lower than that of Ovm-Sil under the same chromatographic conditions.

Fig. 6 shows a three-dimensional chromatogram, from an Ovm-Poly column, of racemic chlorprenaline that had been added to 1 ml of plasma at a concentration of 0.50 μg/ml. The unknown peak that appeared before the peak of (−)-chlorprenaline was also recognized in the chromatogram of blank plasma. The calibration curves were obtained by analysing plasma samples spiked with racemic chlorprenaline in the concentration range from 50 ng/ml to 5 μg/ml. The curve was linear for each enantiomer (correlation coefficient 0.99) and extrapolated plots passed through the origin. The minimum detectable concentration was 50 ng/ml of plasma for racemic chlorprenaline and 25 ng/ml for each chlorprenaline enantiomer. The coefficients of variation (C.V.) for the normalized peak-area ratios were less than 9.0% for each enantiomer. The recoveries were estimated by comparing the peak areas of chromatograms obtained from extracted and standard sample solutions (Table II). Chlorprenaline was observed to be stable during the extraction.

TABLE II

RECOVERY OF CHLORPRENALINE ENANTIOMERS FROM PLASMA

Concentration (ng/ml)	Recovery (mean ± S.D., n = 3) (%)	
	(−)-Chlorprenaline	(+)-Chlorprenaline
50	93 ± 2	90 ± 7
100	92 ± 4	90 ± 6
500	96 ± 4	95 ± 1
1000	99 ± 6	98 ± 4

DISCUSSION

Polymer stationary phases, with such advantages as chemical stability, are useful for affinity and ion-exchange high-performance liquid chromatography (HPLC), for example in regeneration procedures as for open chromatography columns. Recent polymer technology has made possible the development of HPLC stationary phases that have almost the same efficiency as a silica gel column [6]. Asahipak gel is a vinyl alcohol copolymer with good mechanical strength, though its polymeric structure has not been announced. Asahipak gel NH2P-50 introduced polyamine (pentaethylenehexamine) on the gel surface and is now used for the HPLC of sugars [7]. The length of the polyamine is more suitable as the spacer of affinity gel support than is the C_3 of aminopropyl silica gel, and we attempted to increase the chiral resolution ability of ovomucoid by conjugating it to Asahipak gel NH2P-50. The method of conjugating ovomucoid to NH2P-50 was the same as that used in the synthesis of ovomucoid-conjugated silica gel. The amount of ovomucoid conjugated to the polymer was almost the same as that conjugated to the silica gel.

The retention of acidic solutes in Ovm-Poly was much greater than that in Ovm-Sil (Figs. 2–5). The increase of the capacity factor was markedly greater for ketoprofen than for maleic acid. Although the retention of solutes is mainly influenced by the ligand ovomucoid, the small difference in the amount of conjugated ovomucoid suggests that ketoprofen and the polymer support gel interact. The results of the zeta potential determination showed the existence of positive charges resulting from the secondary amines in the spacer region of Ovm-Poly. This feature can be attributed to the increased retention of maleic acid. These results suggest that the characteristics of protein-bonded phases are affected not only by the nature of ligand protein but also by stationary phase support, and Ovm-Poly is useful for the analysis of amines in which acidic contaminants may be present. Compared with Ovm-Sil, Ovm-Poly showed almost the same α but slightly lower N values (Table I). The latter may be partly improved by changing the column-packing technique; however, efforts should be made to develop polymer phases that endure high packing pressure, since this will improve the column efficiency.

The calibration curves on which the peak areas of chlorprenaline enantiomers were plotted showed good linearity; Ovm-Poly columns are applicable for the determination of enantiomer ratios, *e.g.* in pharmaceutical analysis.

Chlorprenaline is an effective β-sympathomimetic agent, only the (−)-form exerting the effect [8]. It is widely used as a bronchodilator, though a survey of the literature revealed no studies on the enantiospecific pharmacokinetics of the compound. The calibration curves of extracts and data in Table II show that plasma levels of chlorprenaline enantiomers are detectable by the proposed method; an enantiospecific pharmacokinetic study will be possible with the Ovm-Poly column, which was easy to regenerate by washing with buffers of 20 mM potassium phosphate (pH 8.5 and pH 3.0) containing 30–50% ethanol.

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