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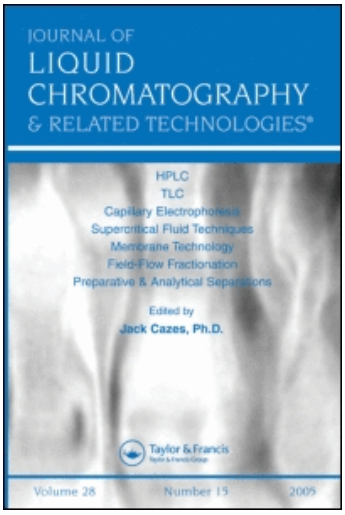

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Retention Characteristics of Protein-Based Chiral HPLC Columns

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RETENTION CHARACTERISTICS OF PROTEIN-BASED CHIRAL HPLC COLUMNS

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ABSTRACT

The retentivity and enantioselectivity of chiral HPLC columns containing immobilized stereoselective proteins as the stationary phase were investigated by varying the parameters of sample size, column temperature, mobile phase pH, mobile phase organic modifier and inorganic buffer concentration. Columns containing bovine serum albumin, ovomucoid, and alpha(1)-acid glycoprotein were evaluated and retention characteristics were compared by separating acidic and basic test compounds. Enantioselectivity of each of these protein-based chiral columns is affected somewhat differently by the chromatographic parameters; chiral separations were optimized by adjustment of the appropriate parameters.

INTRODUCTION

The separation and analysis of chiral compounds has been revolutionized in the last decade by dramatic new developments in high performance liquid chromatography (HPLC). Many new chiral

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HPLC columns with a variety of different stationary phases are now commercially available and are being used in pharmaceutical and agricultural chemical research. An important recent innovation has been the development of chiral HPLC columns that contain immobilized stereoselective proteins as the stationary phase. These proteins are chemically bonded to silica particle supports in HPLC columns to provide remarkable chiral separation capabilities. Stereoselective proteins that have been used as stationary phases include the alpha(1)-acid glycoprotein (1,2), bovine serum albumin (3,4), ovomucoid (5,6), alpha-chymotripsin (7), CBH I cellulase (8), and human serum albumin (9). These protein-based columns can separate the enantiomers of a wide variety of chiral compounds and each of the first three of the above proteins is currently available in commercial columns. While these columns are very useful, they have some unusual retention characteristics. Small changes in column temperature, mobile phase pH, and mobile phase organic modifier often dramatically influence retentivity and chiral resolution.

The sample capacity of these protein-based chiral columns is small (10). Chiral samples of more than 3 nanomoles cause significant decrease in retention and resolution (11,12) although much larger samples can be resolved if the separation conditions have been properly optimized. This small capacity means that these

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protein-based chiral columns generally will be of limited use for preparative isolations. These columns are also not very efficient and generally give broad sample peaks with less than 3500 theoretical plates per 15 cm column (11,12). This combination of small capacity and broad peaks also limits these columns for doing trace analysis since small broad peaks can be difficult to quantitate. Quantitation limits can be increased if the separation conditions are optimized so that larger sample loads can be chromatographed and analyzed.

In this study the retention characteristics and enantioselectivity of three commercially available protein-based chiral columns were investigated and compared. The Chiral AGP, Ultron ES-OVM, and Resolvosil BSA respectively contain immobilized alpha(1)-acid glycoprotein, ovomucoid, and bovine serum albumin as the stereoselective stationary phase. These columns were studied with both an acidic and a basic chiral compound; warfarin and an experimental pharmaceutical compound, DuP 630. They were chosen because they represent typical pharmaceutical compounds and the pure enantiomers of both were available. The retention and resolution of the test compounds on these columns were studied by changing the parameters of sample size, column temperature, mobile phase pH, and mobile phase organic and inorganic buffer concentration. The results on the different columns are compared and examples are given on how separation of the enantiomers was

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maximized by optimization of these parameters. Reference is also made to a recent study in which warfarin was used to test a chiral column that contains a CBH I cellulase protein as the stationary phase(8).

EXPERIMENTAL

Equipment

The studies of the chiral HPLC columns were done with two HPLC systems. A Hewlett-Packard Model 1050 HPLC equipped with a column oven, variable wavelength UV detector, autosampler, and Hewlett-Packard Model 3396 recording integrator (Avondale, PA, USA) was used for study of sample capacity and column temperature. A second system consisting of a Rainin Rabbit HPX model 48-010 pump and a Rainin Dynamax HPLC Macintosh Computer Controller (Woburn, MA, USA), a SSI Lo-Pulse dampener (State College, PA, USA), a Hewlett-Packard Model 1050 autosampler (Avondale, PA, USA), an Applied Biosystems Model 757 variable wavelength UV detector (Foster City, CA, USA), and a Fiatron TC-50 column temperature controller (Oconomowoc, WI, USA) was used for the mobile phase pH, organic modifier and inorganic buffer studies.

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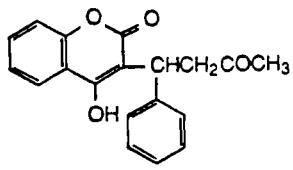
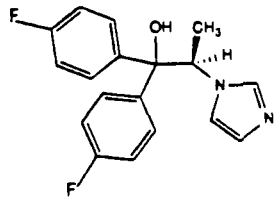
Chemicals

The pure R and S enantiomers of warfarin and DuP 630 (see structures in Figure 1) were obtained from The Du Pont Merck



WARFARIN



DuP 630

Figure 1. Structures of Warfarin and DuP 630

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Pharmaceutical Company in Wilmington, DE, USA. Warfarin is an acidic compound with a pKa of 5.0 and DuP 630 is a basic compound with pKa of 6.8. Both are soluble in isopropanol, acetonitrile, and aqueous mixtures of these solvents. Samples of individual enantiomers were made in concentrations of 0.1 mg/mL in the same solvent mixtures as used for the mobile phases shown in Table 1.

Chiral HPLC Columns

The Chiral AGP column (AGP) from Chromtech in Norsburg, Sweden is 10 cm by 4mm ID in dimension and contains a packing of alpha(1)-acid glycoprotein immobilized onto silica particles by a

covalent linkage and cross-linking of the protein molecules. The alpha(1)-acid glycoprotein contains 181 amino acids and 5 carbohydrate units and has a molecular weight of approximately 141,000 daltons and an isoelectric point of 2.7 (2). The manufacturer recommends that the columns be used in the mobile phase pH range of 3 - 7 with up to 25% of organic modifier (isopropanol, acetonitrile, etc). The column has been used at temperatures up to 70° C (2) and can separate a variety of acidic and basic chiral compounds.

The Resolvosil BSA-7 column (BSA) from Machery Nagel GmbH in Duren, FRG is 15cm by 4 mm ID and contains bovine serum albumin covalently bonded to silica particles. The bovine serum albumin is a globular protein consisting of 581 amino acids with a molecular weight of 66,210 daltons and an isoelectric point of 4.7 (4). The column can be used in the pH range of 5 - 9 with up to 5 - 10% of organic modifier such as n-propanol. Since this column does not separate the enantiomers of basic chiral compounds such as DuP 630, it was used only for the study of the warfarin enantiomers.

The Ultron ES-OVM column (OVM) from Shinwa Chemical Industries in Kyoto, Japan is 15 cm by 4.6 mm ID and contains a packing of ovomucoid protein covalently bonded to a silica particle support. Ovomucoid is a stable glycoprotein obtained from egg white with a molecular weight of approximately 28,800 and isoelectric point of 3.7 - 4.5 (13). The manufacturer recommends that the OVM

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column be used in the pH range of 3 - 7.5 with up to 50% of an organic modifier in the mobile phase and at column temperatures up to 40° C. The OVM column can separate both acidic and basic chiral compounds

Chromatographic Conditions

Similar chromatographic conditions were used to separate the enantiomers of the two test compounds so that the retention behavior of the columns could be more closely compared. Phosphate buffers were prepared from 5, 10, 20, or 30 mM sodium phosphate solutions which were adjusted to the desired pH with 1 molar sodium hydroxide or phosphoric acid. Isopropanol (IPA) was chosen as the organic modifier since it gave the best overall separation of the enantiomers on the various columns. The IPA concentration was adjusted to give comparable retention of the test compounds on each column and the two sets of similar chromatographic conditions shown in Table 1 were used for sample capacity studies. HPLC conditions A were used to separate warfarin on the AGP column and DuP 630 on the AGP and OVM columns; samples of warfarin were studied on the BSA and OVM columns with HPLC conditions B. HPLC conditions A and B were also used in the studies of temperature, mobile phase pH, organic modifier concentration, and inorganic buffer concentration except that each parameter was individually varied in its respective study.

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TABLE 1
HPLC CONDITIONS

	A.	B.
Mobile Phase	85% aqueous buffer* 15% IPA	90% aqueous buffer* 10% IPA
Temperature	30 C	30 C
Flow Rate	0.9 mL/min	0.9 mL/min
Detector	UV 280 nm (warfarin) 230 nm (DuP 630)	UV 280 nm
Sample	0.5 micrograms of each enantiomer	

*10mM sodium phosphate pH 6.5

Calculations

Chromatographic calculations of capacity factor (k'), selectivity factor (α), resolution factor (Rs), and theoretical plate number (N) were performed according to Snyder and Kirkland (14).

RESULTS AND DISCUSSION

Sample Capacity

The effect of sample size on retention and selectivity of the three chiral columns was investigated by chromatographing 0.8, 1.6, 3.0, 8.0, 16.0, and 30.0 nanomole samples of the individual enantiomers. Individual sample solutions of 0.01, 0.02, 0.05, and 0.10 mg/mL of each enatiomer were made in the mobile phase used for each study and appropriate volumes were injected to give the desired sample size. The chromatographic conditions used in these studies are listed in Table 1. The capacity factor and efficiency (in theoretical plates) of the first eluting enantiomer were measured and are listed in Table 2. Significant decreases in retention and column efficiency were seen for individual enantiomer samples of greater than 3 nanomoles (approximately 1 microgram) for all samples and columns studied. This limited sample capacity has previously been noted on protein-based chiral HPLC columns and presumably results from the small amounts of protein which are bonded to the

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TABLE 2

Effect of Sample Size

	Warfarin						DuP 630			
Nano	AGP		OVM		BSA		AGP		OVM	
moles	k'	N	k'	N	k'	N	k'	N	k'	N
0.8	2.22	500	2.18	1340	19.1	580	4.63	1120	1.87	3234
1.6	2.21	480	2.17	1340	18.9	580	4.60	1120	1.82	3385
3.0	2.15	460	2.10	1070	18.8	521	4.47	1040	1.79	2644
8.0	1.96	340	2.04	750	17.8	428	4.43	770	1.75	2244
16.0	1.80	220	1.99	630	17.3	345	4.26	690	1.71	1870
30.0	1.56	150	1.95	610	16.3	264	4.14	530	1.69	1416

chromatographic supports (10). This limited sample capacity also caused a dramatic decrease in resolution with increase in sample size as shown in the graph in Figure 2.

Although column performance deteriorates for samples greater than 3 nanomoles, much larger samples can be chromatographed on these protein-based chiral columns. Sample loads of more than 30 micrograms may be baseline separated for enantiomers that are initially well resolved (Rs > 3 for small samples). The resolution of enantiomers may often be greatly increased by optimizing the separation. This can be done by adjusting the chromatographic parameters of column temperature, mobile phase pH, organic modifier concentration, and inorganic buffer concentration. The effect of these parameters on retention and enantioselectivity is discussed in the following sections.

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Effect of Column Temperature

The retentivity of protein-based chiral HPLC columns is strongly affected by temperature as shown in Table 3. A 10° C increase in temperature can cause a 20 to 30% decrease in retention. The results in Table 3 also show that the enantioselectivities of these chiral separations are slightly decreased as the temperature is increased. This results in a slight decrease in resolution with increasing temperature which is typical of chiral separations on protein-based chiral columns (2,16,18). However, these

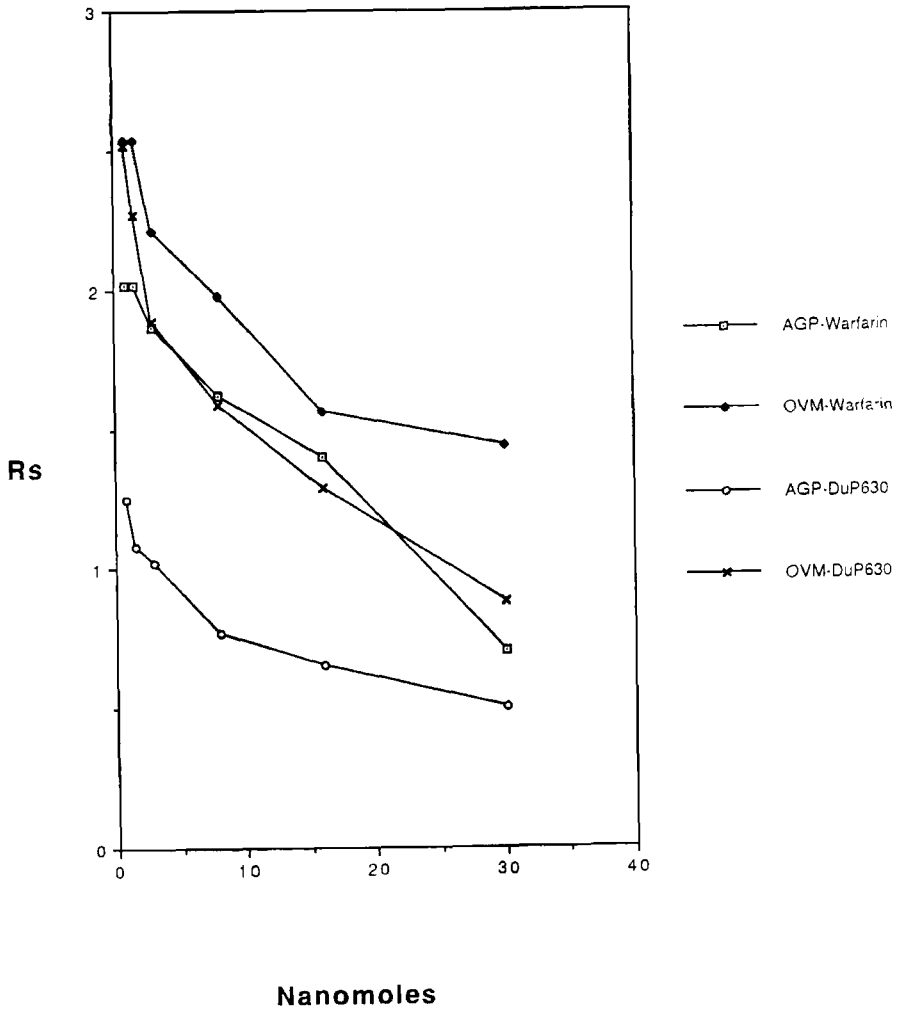

Figure 2. Effect of Sample Size on Resolution Factor

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TABLE 3

Effect of Column Temperature

	Warfarin						DuP 630			
	AGP		OVM		BSA		AGP		OVM	
C	k'	α	k'	α	k'	α	k'	α	k'	α
25	2.63	1.67	2.33	1.48	21.5	1.13	5.38	1.23	2.11	1.84
30	2.30	1.64	2.18	1.40	19.1	1.12	4.67	1.21	1.94	1.79
35	1.97	1.61	1.97	1.36	16.5	1.10	4.02	1.19	1.69	1.68
40	1.54	1.54	1.75	1.31	15.1	1.09	3.37	1.17	1.43	1.62
45	-	-	1.57	1.28	-	-	-	-	1.27	1.50

enantioselectivity changes are generally small and temperature can be used as a parameter to adjust the retention time of a separation without significantly affecting resolution.

Effect of Organic Modifier

The mobile phase used with protein-based chiral columns can be modified with uncharged organic solvents, such as isopropanol or acetonitrile. These organic modifiers can have a dramatic effect on column selectivity as shown in the chromatograms in Figure 3 where a change from 15% acetonitrile to 15% isopropanol in the mobile phase gave better resolution and reversed the elution order of the enantiomers of warfarin on the AGP column. A similar reversal of elution order does not occur on the OVM or BSA column and is unusual since it has been observed in only a few other separations (11,15). Although many organic solvents can be used as mobile phase modifiers (11,17), isopropanol and acetonitrile appear to be the most widely used and give the greatest difference in enantioselectivity.

The concentration of an organic modifier can also strongly affect the protein-based chiral columns as shown in Table 4 where the capacity factor of the first eluting enantiomer and the selectivity factor for both enantiomers is listed. A relatively small decrease in isopropanol concentration (from 20 to 10%) can cause up to a tenfold

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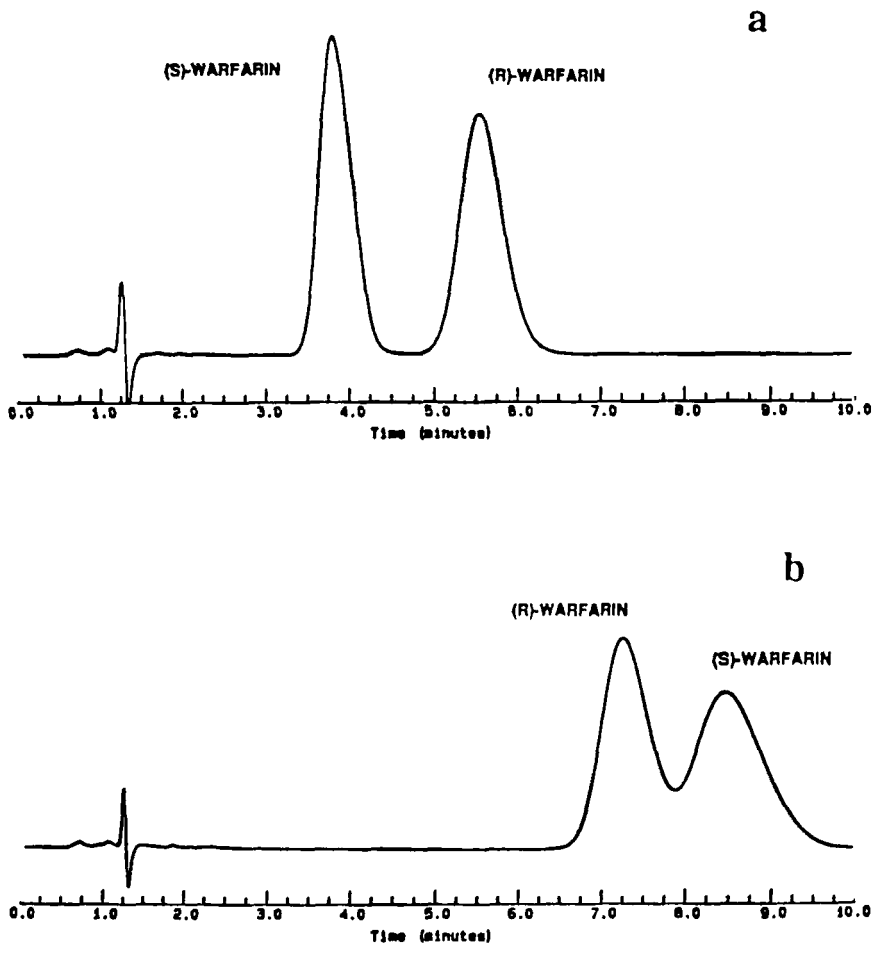

Figure 3. Separation of Warfarin Enantiomers on AGP Column with; (a) HPLC conditions A in Table 1 with 15% isopropanol modifier, (b) HPLC conditions A but with 15% acetonitrile modifier.

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TABLE 4
Effect of Mobile Phase Isopropanol Concentration

	Warfarin						DuP 630			
%IPA	AGP		OVM		BSA		AGP		OVM	
	k'	α	k'	α	k'	α	k'	α	k'	α
2.5	-	-	-	-	63.0	1.22	-	-	-	-
5	-	-	4.53	2.23	37.8	1.19	-	-	-	-
10	7.20	1.63	2.08	1.42	19.1	1.12	8.20	1.43	3.85	1.92
15	2.30	1.63	1.23	1.17	-	-	4.20	1.31	1.90	1.65
20	0.71	1.71	0.84	1.02	-	-	2.60	1.23	1.56	1.43
25	0.24	1.70	-	-	-	-	1.40	1.21	1.07	1.30

increase in retention and may dramatically increase enantioselectivity. The enantioselectivity of the OVM column is much more strongly affected by change in IPA concentration as compared to the AGP and BSA columns. This is illustrated in Figure 4 where a small change in IPA concentration caused complete separation of unresolved warfarin enantiomers on the OVM column. It is interesting that a literature study of an experimental chiral column with the CBH I cellulase stationary phase (8) found the opposite effect to that in Table 4; an increase in organic solvent concentration tended to significantly increase enantioselectivity of that column.

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Charged organic modifiers, such as octanoic acid or dimethyloctylamine, have a very strong effect on compound retention and peak shape on these protein-based chiral columns (2,18,21). Relatively small amounts (less than 1%) of acid and base modifiers may sometimes dramatically influence enantioselectivity. However, these charged organic modifiers may be difficult to remove from the column because of their affinity to the protein stationary phase (19). For this reason an investigation of the effect of charged modifier was not pursued in this study.

Effect of Mobile Phase pH

The protein-based chiral columns are used in a mobile phase pH range which is limited by the stability of the protein and the

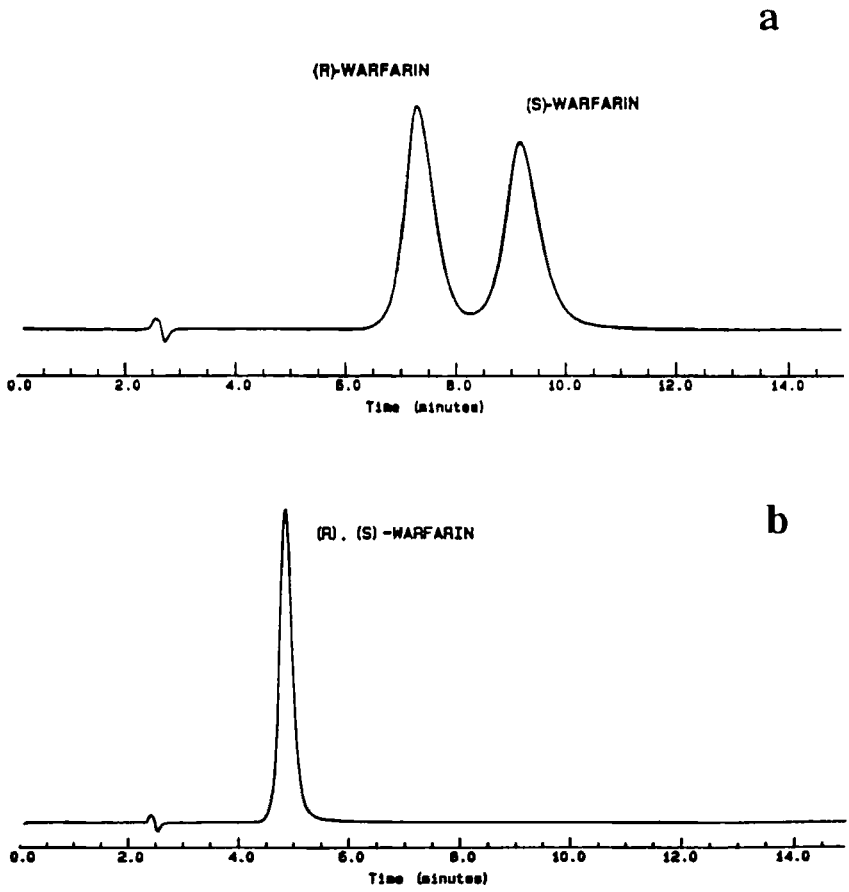

Figure 4. Separation of Warfarin Enantiomers on Ultron ES-OVM column with; (a) HPLC conditions B in Table 1 with 10% isopropanol modifier, (b) HPLC conditions B but with 20% isopropanol modifier.

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silica support used for the stationary phase. The pH in the range from 4 to 7 appears to be most useful and pH change has a strong affect on retentivity of these chiral columns as shown in Table 5. The retention of acidic compounds increases and basic compounds decreases with decreasing pH (5,7,12). Other studies have shown that the retention of neutral chiral compounds is almost unaffected by pH change (2,7,20).

The enantioselectivity of the AGP and OVM columns can be significantly affected by pH as shown by the data in Table 5 and in other studies (7,18). Small changes in mobile phase pH can sometimes cause dramatic change in separation of unresolved enantiomers as illustrated in Figure 5. Better enantioselectivity is generally obtained on these protein-based chiral columns if acidic compounds are separated at mobile phase pH of less than 6 and basic compounds at pH greater than 6.

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The dramatic changes in enantioselectivity with change in mobile phase pH indicate that the AGP and OVM columns have protein stationary phases with multiple stereoselective binding sites (21). If only one stereoselective site were involved in binding of both enantiomers, then retentivity of both should have been similarly affected by pH change and enantioselectivity would not have changed so significantly. The difference in elution order of warfarin enantiomers on the AGP and OVM columns (compare

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TABLE 5
Effect of Mobile Phase pH

	Warfarin						DuP 630			
pH	AGP		OVM		BSA		AGP		OVM	
	k'	α	k'	α	k'	α	k'	α	k'	α
4.0	16.70	2.44	6.88	2.19	50.5	1.11	0.17	1.03	0.15	1.10
5.0	13.85	2.06	6.37	2.00	31.4	1.11	1.40	1.06	0.73	1.30
6.0	4.90	1.81	3.65	1.53	21.4	1.11	4.31	1.18	1.42	1.51
7.0	1.14	1.68	1.28	1.26	4.6	1.12	4.73	1.40	2.71	1.86

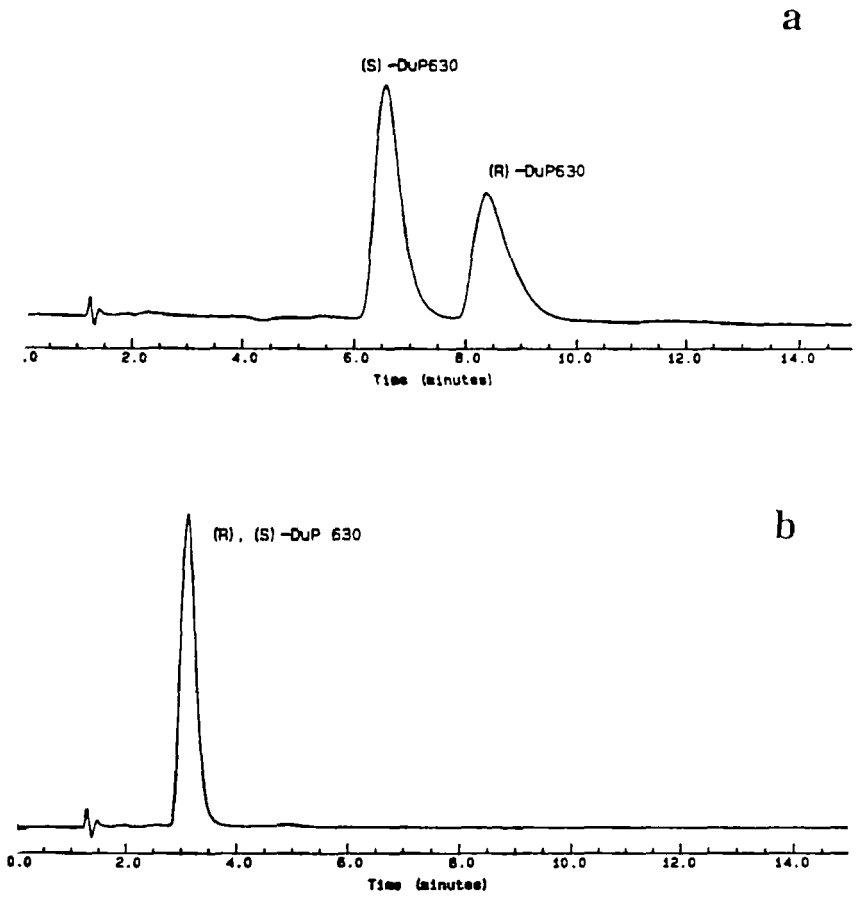

Figure 5. Separation of DuP 630 Enantiomers on Chiral AGP column with; (a) HPLC conditions A in Table 1 with pH 6.5,
(b) HPLC conditions A but with pH 5.0.

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TABLE 6

Effect of Mobile Phase Inorganic Buffer Concentration

	Warfarin						DuP 630			
Milli-	AGP		OVM		BSA		AGP		OVM	
molar	k'	α	k'	α	k'	α	k'	α	k'	α
5	2.04	1.76	2.03	1.32	22.3	1.12	6.47	1.20	2.03	1.39
10	2.28	1.72	2.03	1.32	19.1	1.12	4.58	1.24	1.98	1.38
20	2.84	1.65	2.10	1.31	16.2	1.13	3.83	1.27	2.01	1.37
30	3.01	1.61	2.00	1.33	13.2	1.10	3.42	1.32	1.99	1.39

Figures 3 and 4) also indicates that the structures of the multiple binding sites in these two columns are quite different.

Effect of Inorganic Buffer

The concentration of the inorganic buffer generally has a minor effect on the enantioselectivity of protein-based chiral columns (2,11). However, the data in Table 6 show that buffer concentration can significantly affect retention on the AGP and BSA columns and this parameter may be used to adjust their retentivity.

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CONCLUSIONS

Chiral HPLC columns that contain bovine serum albumin (BSA), ovomucoid (OVM), and alpha(1)-acid glycoprotein (AGP) as the stereoselective stationary phase can be used to resolve the enantiomers of a wide range of chiral compounds. The retentivities of all three columns are strongly affected by the mobile phase parameters of pH, temperature, and organic modifier concentration; the inorganic buffer concentration affects retention on the AGP and BSA columns. Enantioselectivities of all three columns are strongly affected by the type of organic modifier used in the mobile phase; the enantioselectivity of the OVM column is also affected by the concentration of the organic modifier. The mobile phase pH most strongly affects enantioselectivity of the AGP and OVM columns

which indicates that their protein stationary phases have multiple stereoselective binding sites. These four mobile phase parameters can be used in combination to control retention and optimize resolution on these protein-based chiral columns.

Protein-based chiral HPLC columns have limited sample capacity which is a disadvantage in preparative isolation of pure enantiomers and is also a limitation when separating and measuring trace chiral components such as the distomer impurity in a single enantiomer pharmaceutical compound. However, larger chiral sample loads can be chromatographed and resolved if separation conditions are properly optimized. Since the interaction of column temperature, mobile phase pH, organic modifier, and inorganic buffer concentration can affect retention and enantioselectivity, all should be considered when determining the conditions for optimum resolution. Experimental design strategies based on multivariate analysis techniques are now being developed to simplify this optimization process (22,23) and will be the subject of further studies and publications.

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