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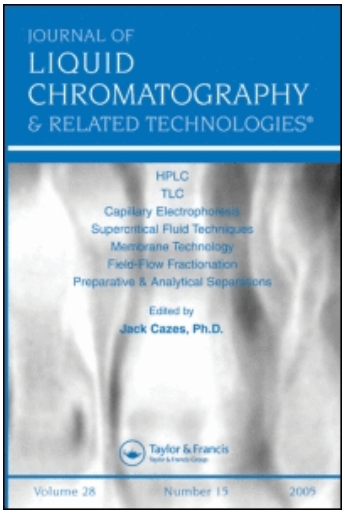

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CHIRAL SEPARATIONS OF SELECTED PHARMACEUTICALS ON AVIDIN COLUMN

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ABSTRACT

An unexplored commercially available avidin protein column was investigated for chiral separation of selected pharmaceuticals. Mobile phase compositions such as pH, buffer strength, and organic modifier were varied to affect separation of racemic mixtures. Baseline resolution of enantiomers were successfully achieved for thalidomide, glutethimide, primaquine, aminoglutethimide, hydroxyzine, chlorthalidone, and pyridoglutethimide. Partial separation of enantiomers were achieved for oxazepam, lorazepam, verapamil, and hexobarbital. Separation conditions of each racemate were optimized so that the methods can be used for the analysis of racemic pharmaceuticals. The pH and organic modifier of mobile phase played a greater role than buffer strength in the separation of individual racemate. The presence of aromatic rings and carbonyl functions directly attached to the chiral center of an analyte may play an important role in recognizing a specific site in the protein bonded phase.

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INTRODUCTION

It is well recognized that enantiomers can have different pharmacological activity in humans.[1,2] The importance of administering a single enantiomer in therapeutics has been reviewed.[3,4] To understand the pharmacology/ pharmacokinetics and toxicology of the enantiomers, stereoselective assay methods are needed for their quantitation in serum/plasma or other body fluids.Today, chiral separations in HPLC are performed mainly by direct and indirect methods. Most earlier works were done by indirect method by chemical derivatization of the enantiomers to diastereoisomers. Since the early eighties, the direct method of chiral separation by HPLC has enjoyed popularity because of its simplicity. Direct chiral separation is performed by either employing a chiral stationary phase(CSP) or employing a mobile phase containing a chiral selector. Direct chiral separation with a chiral column is more popular than using a chiral selector in the mobile phase because the chiral selector may be costly and can interfere with peak/s of interest at low UV wavelength.

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There are four major classes of chiral columns available commercially: protein, cyclodextrin, carbohydrate, and pirkle phases.[5-7] Two other columns which are commercially available but have not found much applications are ligand exchange and silica based bonded macrocyclic antibiotic column.[8] Several review articles have described the mechanism of separation on these different columns and suggested which column is best suited to certain types of analytes.[9,10] Some CSPs are more universal than the others to separate analytes of widely different structures. Protein columns are found to be more universal than carbohydrate columns followed by pirkle and cyclodextrin columns. It was found that racemates, which are difficult to separate with other CSPs, can be successfully resolved into enantiomers on protein columns.[11]

Although, in the scientific literature, construction of many protein stationary phases has been elaborated, only seven columns with somewhat different enantioselectivity have been commercialized. The commercial CSPs are bovine and human serum albumin, alpha 1-acid glycoprotein, ovomucoid, cellobiohydrolase (cellulase), pepsin, and avidin. Proteins in these columns are immobilized on wide pore silica or polymeric support material by covalent bonding with a hydrophobic spacer. A special advantage of these protein columns is that they are compatible with aqueous buffer systems widely used in biological applications. Protein columns are less suitable for preparative applications because of their limited sample capacity.

The avidin protein is covalently bonded to the silica surface by a hydrophobic spacer. Oda et al.[12] has investigated the retention behavior of racemic drugs on avidin and modified avidin columns. Haginaka et al.[13] has studied the retention behavior and enantioselectivity of 2-arylpropionic acid derivatives on an avidin column. They have also investigated the effect of base

material, spacer type, and protein modification on retention and chiral recognition of 2-arylpropionic acids. Mano et al.[14] has compared the retention behavior of avidin protein phase with ovomucoid, conalbumin, and flavoprotein phases. Avidin column was shown to have an excellent enantioselectivity for 2-arylpropionic acids.

So far, the avidin column has found very few applications in chiral separation. To date, only enantiomers of arylpropionic acids, chlormezanone, cloperastine, and trihexyphenidyl have been shown to separate on the avidin column.[12,13,14] The objective of this paper was to explore new applications for this protein column and to monitor the performance of this column over time.

EXPERIMENTAL

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Reagents and Chemicals

Thalidomide, aminoglutethimide, and their enantiomers were purchased from Research Biochemical Incorporated (Natick, MA). Chlorthalidone, hydroxyzine hydrochloride, and lorazepam were obtained from USP (Rockville, MD). Glutethimide, oxazepam, ketoprofen, calcium fenoprofen, ibuprofen, primaquine, hexobarbital, and verapamil were purchased from Sigma (St Louis, MO). Ketoprofen and pyridoglutethimide enantiomers were donated by Rhone Poulenc (France) and US Bioscience (West Conshohocken, PA), respectively. All solvents were of HPLC grades and the reagents were of highest quality commercially available.

Freshly distilled water was used in mobile phase preparation. The Bioptic AV-1 column (150 mm x 4.6 mm i.d.) and its guard column were obtained from Meta Chem Technologies (Torrance, CA).

Instrumentation and Chromatographic Condition

The HPLC system consists of a Beckman pump (Model 110A), an Alcott autosampler (Model 278) equipped with a 100 μL loop and a UV/Vis detector (Waters, Model 481). The chromatograms were recorded on a Shimadzu integrator (Model C-R3A, Chromatopac). All the separations were performed on the Bioptic column at a flow rate of 0.6 mL/min and at ambient temperature (22±1°C). The column was protected with a guard column containing the same stationary phase and a frit filter coupled on line. The detection wavelength was set at 254 nm.

Preparation of Standards

Stock solutions of 1000 μg/mL of each standard were prepared in methanol except hydroxyzine hydrochloride, which was prepared in distilled water. All stock solutions were stored at 4°C. Aliquots from each stock were spiked into 1 mL of distilled water to make the required concentrations, vortexed, and injected into the HPLC system.

RESULTS AND DISCUSSION

Avidin is a basic protein having an isoelectric point between 9.5 to 10. The protein contains functional groups such as histidine, tryptophan, aspartic acid, and glutamic acid. The ionization state of the functional groups depends on the pH (2.5-7.5)of the mobile phase. Retention of drugs on the avidin column depends on pH, ionic strength, type of organic modifier and anions in the mobile phase. Retention of an acidic drug on an avidin column usually increases as the pH is decreased because of increased hydrophobicity of the analyte. In some cases, retention of an acidic drug may increase as the pH is increased if the electrostatic interaction predominates for the analyte. The pH effect is reversed for an amine type drug. The effect of ionic strength is more complex because both electrostatic and hydrophobic interactions come into play. Retention of the uncharged molecule on the avidin column is less affected by the pH and buffer strength of the mobile phase. With aqueous buffer as mobile phase, the retention time of a hydrophobic drug can be very long. The addition of organic modifier in the mobile phase reduces the retention time and improves the peak shape of an analyte.

The resolution of racemic mixtures is strongly influenced by the pH and organic modifiers contained in the mobile phase. The buffer type and strength also contribute to chiral recognition, but to a lesser extent. The exact mechanism of chiral recognition in the avidin column has not been elucidated, but it is generally believed that both electrostatic and hydrophobic interaction are contributors. It is also believed that nonspecific forces such as charge transfer interaction and hydrogen bonding are involved in the enantioselectivity. The multiplicity of the binding interactions affects the net affinity of the enantiomers for the protein to be highly dependent on steric factors. The steric contributions are different for two enantiomers in contact with a protein surface, which leads to a difference in retention times and thereby resolution of the enantiomers. Eleven racemic mixtures were selected for study on the avidin column. Baseline resolution of enantiomers was successfully achieved for thalidomide, glutethimide, primaquine, aminoglutethimide, hydroxyzine, chlorthalidone, and pyridoglutethimide.

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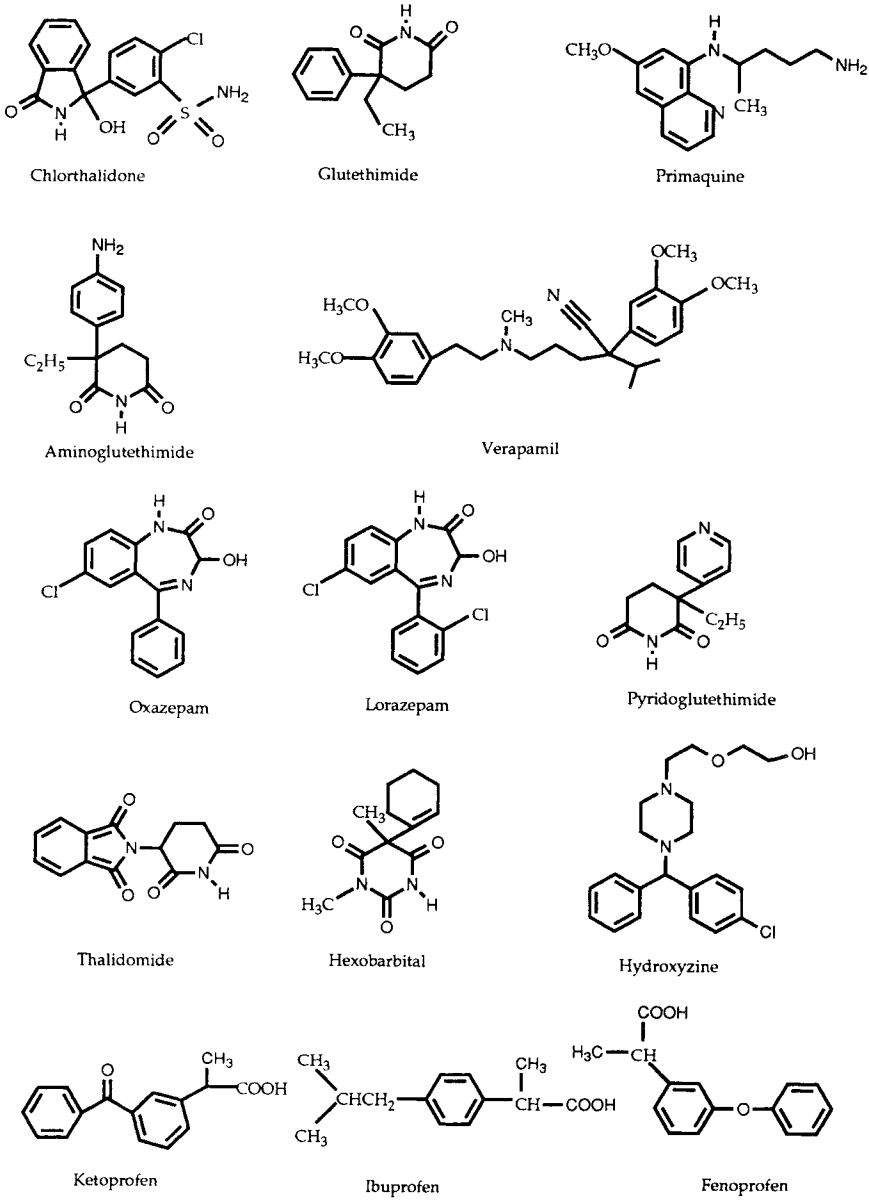

Figure 1. Chemical structures of racemic drugs studied.

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Partial separation of enantiomers was achieved for oxazepam, lorazepam, verapamil, and hexobarbital. Chemical structures of the pharmaceuticals studied are shown in Fig 1. To effect separation of the enantiomers, pH (2.5-7.5), buffer strength (0.05-0.15M) and organic modifier (1-10%) concentration of the mobile phase were manipulated. All chromatographic separations were performed at the flow rate of 0.6 mL/min at ambient temperature with detection at 254nm. The effect of mobile phase conditions on the separation of each racemic mixture on the avidin column are described below.

Retention of chlorthalidone enantiomers on avidin column was not affected by the change of pH and buffer strength of the mobile phase. This was expected because chlorthalidone (pKa 9.4) remains mostly unionized in the pH range studied. The enantiomers remain separated at the pHs (2.5-7.5) studied. Methanol gave the largest separation factor and resolution compared to either 2-propanol or acetonitrile. Although the resolution was excellent with methanol, the total chromatographic run time was longer. The separation conditions were optimized with acetonitrile because the chromatographic run time was shorter and resolution was equally good to that of methanol. Retention of this compound on the avidin column depends on the hydrophobic and other nonspecific interactions with the stationary phase. The separation (Fig 2A) of chlorthalidone enantiomers was achieved with a mobile phase of 4:96 v/v acetonitrile- phosphate buffer(pH 4, 0.1 M).

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The retention factor of glutethimide on the avidin column did not change significantly at various pHs and buffer strengths of the mobile phase. This is because glutethimide (pka 11) remains mostly unionized in the pH range studied. The addition of organic modifier in the mobile phase plays a very important role in the resolution of enantiomers. It was not possible to achieve baseline separation of the enantiomers with only aqueous buffer (pH 2.5-7) as the mobile phase. Addition of organic modifiers in the mobile phase effects baseline separation of the enantiomers. Among the organic modifiers investigated, methanol gave the best separation factor between enantiomers compared to acetonitrile or 2-propanol. Retention of this drug on the avidin column depends on hydrophobic and other nonspecific interactions. The separation (Fig 2B) of glutethimide enantiomers was achieved with a mobile phase of 4:96 v/v methanol-phosphate buffer (pH 2.5, 0.1M).

The retention factor of the primaquine on the avidin column was strongly affected by the pH of the mobile phase. The retention time increased as the pH of the mobile phase increased. Buffer concentration of the mobile phase did not significantly change the retention time. The resolution of the enantiomers was affected by the pH of the mobile phase. Primaquine, classified as a weak base, remains mostly protonated in the pH range studied.

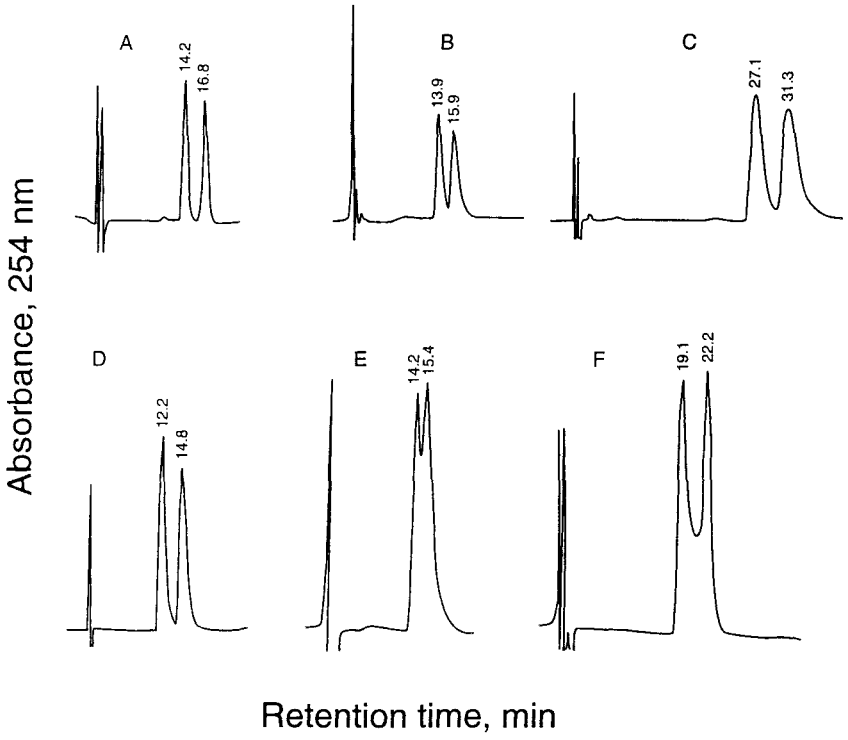

Figure 2. Representative chromatograms of racemic drugs on avidin column with optimized mobile phases at a flow rate of 0.6 mL/min and ambient temperature. A) Chlorothalidone B) Glutethimide C)Primaquine D) Aminoglutethimide E) Verapamil F) Oxazepam. Mobile Phase Conditions: See Table 1.

At pH below 3.5, the resolution of the enantiomers decreased. It is likely that above pH 3.5, the positively charged primaquine undergoes electrostatic interaction with negatively charged amino acids on the stationary phase and this columbic interaction plays a significant role in the chiral recognition. Aspartic acid and glutamic acid of the stationary phase remain mostly undissociated below pH 3.5. Among the three solvents tested, 2-propanol gave the best resolution of the enantiomers. The primaquine enantiomers were separated (Fig 2C) with a mobile phase of 3: 97 v/v 2-propanol-buffer (pH 6, 0.1M).

The retention of aminoglutethimide enantiomers on the avidin column was strongly affected by the pH of the mobile phase. The racemic mixture was separated (Fig 2D) with a mobile phase of aqueous phosphate buffer (0.1M, pH6). The retention time of the drug increased as the pH increased because of

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increased hydrophobic interaction. Below pH 4, the resolution of the enantiomers decreased. This may be due to protonation of the amine function below pH4 and decrease of hydrophobic interaction with chiral recognition cavity.

Verapamil enantiomers were partially resolved (Fig 2E) with a mobile phase of 2:98 methanol:buffer (pH 2.5, 0.1M). The retention factor of the drug increased with an increase in pH. It was not possible to achieve baseline separation of the enantiomers in the pH 2.5 -7.5 range. Among the three solvents investigated, methanol gave the best resolution compared to either acetonitrile or 2-propanol. The buffer concentration of the mobile phase did not affect the resolution of the enantiomers.

The retention factors of oxazepam (pKa 11.1) and lorazepam (pKa 11.5) on the avidin column were not affected by the pH of the mobile phase because the benzodiazepines remain essentially unionized in the pH range studied. The effect of organic solvents such acetonitrile and 2-propanol on the retention of these two drugs was very similar. The benzodiazepines were partially separated with a mobile phase of 4:96 v/v 2-propanol-buffer (pH 2.5, 0.1M). Among the three solvents tested, 2- propanol provided the best resolution of the enantiomers. Retention times of the benzodiazepines (Fig 2F and 3A) increased as the concentration of the buffer in the mobile phase increased.

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The effect of mobile phase composition on the resolution of pyridoglutethimide enantiomers was studied. The retention of the drug increased as pH of the mobile phase increased. Below pH 4, the resolution of the enantiomers decreased. This may be due to protonation of the analyte below pH 4 and decreased hydrophobicity of the drug molecule. The hydrophobic interaction probably plays a significant role in the chiral recognition. 2-Propanol provided the best resolution of the enantiomers compared to either methanol or acetonitrile. The enantiomers of the drug were separated (Fig 3B) with a mobile phase of aqueous phosphate buffer (0.1M, pH 7.5) at ambient temperature.

The separation (Fig 3C) of thalidomide enantiomers was achieved with a mobile phase of 2: 98 v/v 2-propanol-buffer (pH 4, 0.1M). Since thalidomide remains mostly in the unionized state in the pH range studied, the retention of thalidomide on the avidin column was not influenced either by pH or buffer concentration of the mobile phase. The retention of thalidomide probably depends on hydrophobic and other nonspecific interactions with the stationary phase. The resolution of the enantiomers did not change significantly in the pH 2.5-7.5 range. The effect of organic solvent was also studied. Methanol gave the best resolution, but 2-propanol and acetonitrile afforded equally good resolution with shorter retention times.

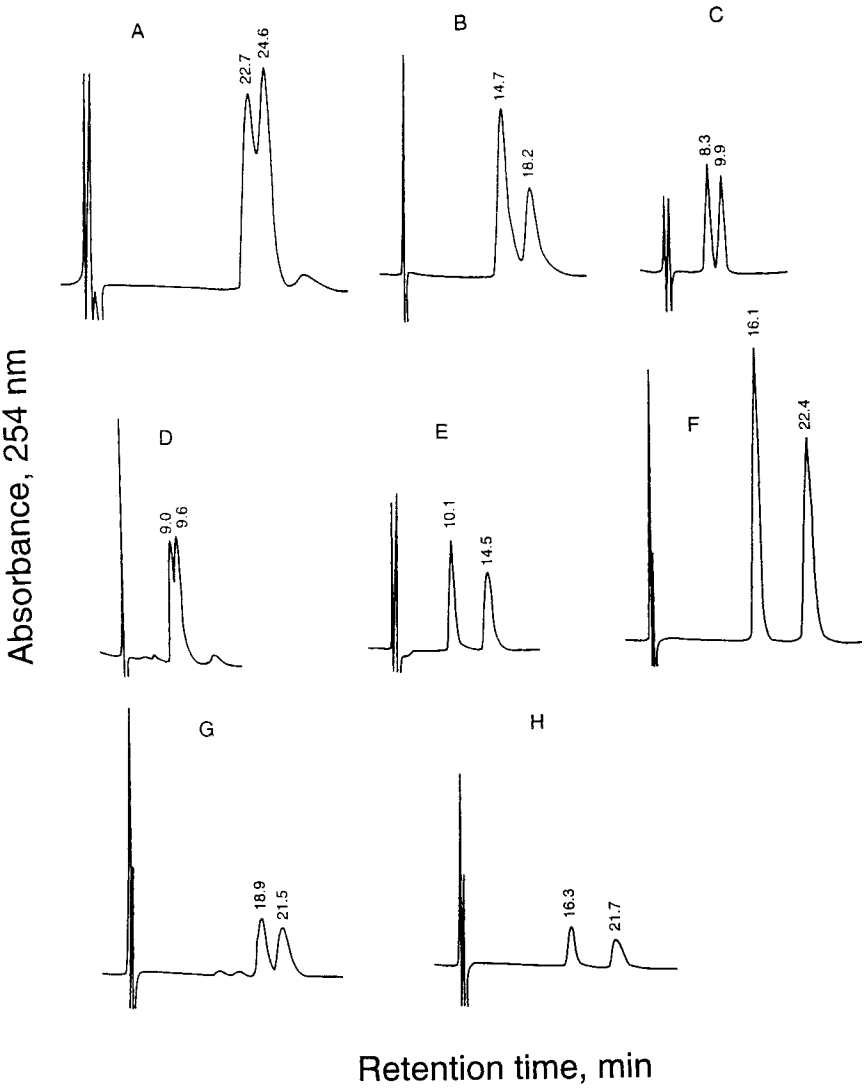

Figure 3. Representative chromatograms of racemic drugs on avidin column with optimized mobile phases at a flow rate of 0.6 mL/min and ambient temperature. A) Lorazepam B)Pyridoglutethimide C)Thalidomide D)Hexobarbital E)Hydroxyzine F) Ketoprofen G) Ibuprofen H) Fenoprofen. Mobile phase conditions: A-E, see Table 1. F-H, see text.

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Hexobarbital was partially separated (Fig 3D) with a mobile phase of phosphate buffer (pH 7.5, 0.1M). The drug remains mostly undissociated in the pH range studied and its retention on the avidin column was not influenced either by pH or buffer concentration of the mobile phase. The retention time decreased with the addition of organic modifier in the mobile phase.

The retention of hydroxyzine on the avidin column was influenced by pH, organic modifier and buffer strength of the mobile phase. The increase of buffer concentration in the mobile phase also increased retention time. Retention of the drug increased at alkaline pH. Acetonitrile provided the best resolution of the enantiomers compared to either methanol or 2-propanol. The separation (Fig 3E) of the enantiomers was achieved with a mobile phase of 4: 96 v/v acetonitrile-buffer (pH 4, 0.1M) at ambient temperature.

Racemic mixtures of ketoprofen (Fig 3F), ibuprofen (Fig 3G) and fenoprofen (Fig 3H) were separated on the commercially available avidin column in this laboratory with a mobile phase of 5:95 v/v acetonitrile-buffer (pH 7, 0.1M) at flow rate of 0.6 mL/min and at ambient temperature. The retention behavior and resolution of these 2-arylpropionic acid derivatives on an avidin phase prepared in the laboratories of Oda, Haginaka and Mano[12-14] were comparable to that obtained in our study. The retention factors, separation factors, and resolution values obtained with each racemic mixture with optimized mobile phases are shown in Table 1.

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This study revealed that both columbic and hydrophobic interaction play a significant role in the retention and chiral recognition of a drug by the avidin protein phase. Upon analyzing our data, a few general remarks can be made about retention and enantioselectivity on the avidin column. Methanol usually provided the largest capacity factor compare to either 2-propanol, ethanol or acetonitrile. Similar retention factors of the drug were observed with either 2-propanol or ethanol in the mobile phase. Acetonitrile gave the shortest retention time with excellent peak shapes compared to other organic modifiers. The pH of the mobile phase was extremely important for both retention and chiral recognition. The buffer concentration of the mobile phase usually plays a less important role in the chiral separation. Mano et al.[14] has reported that the anions of the mobile phase also affect the enantioselectivity on the avidin protein phase. The ability of the analyte and CSP to form transient diastereomeric complexes utilizing hydrogen bonding, pi-pi interactions and dipole stacking is well recognized.[15] It was proposed that a minimum of three simultaneous interactions between analyte and CSP are needed for chiral recognition. [16] All the enantiomers separated in our studies and other investigators[12-14] on the avidin column have either a aryl ring or carbonyl function directly attached to the chiral center. Possibly, the aryl and carbonyl functions undergo hydrophobic and other nonspecific interactions (e.g hydrogen bonding), respectively, with the chiral cavity or chiral recognition site.

Table 1

Retention Factors,[a] Separation Factors and Resolution Values Obtained for Selected Racemic Drugs on Avidin Column with Optimized Mobile Phases

Analyte	k1	k2	α	Rs	Mobile Phase Composition
Chlorthalidone	4.0	5.0	1.25	1.96	4:96 v/v acetonitrile-buffer (pH 7.3, 0.1M)
Glutethimide	4.3	5.1	1.18	1.20	4:96 v/v methanol-buffer (pH 2.5, 0.1M)
Primaquine	8.0	9.4	1.17	1.25	3.97 v/v 2-propanol-buffer (pH 6, 0.1M)
Amino-glutethimide	3.0	3.9	1.30	1.86	Aqueous phosphate buffer (pH 6, 0.1M)
Verapamil	4.4	4.9	1.10	0.70	2:98 v/v methanol-buffer (pH 2.5, 0.1M)
Oxazepam	7.2	8.5	1.18	0.90	4:96 v/v 2-propanol-buffer (pH 2.5, 0.1M)
Lorazepam	8.5	9.6	1.12	0.80	4:96 v/v 2-propanol-buffer (pH 2.5, 0.1M)
Pryido-glutethimide	4.1	5.2	1.20	1.55	Aqueous phosphate buffer (pH 7.5, 0.1M)
Thalidomide	1.9	2.5	1.27	1.11	2:98 v/v 2-propanol (pH 7, 0.1M)
Hexobarbital	2.1	2.3	1.10	0.80	Aqueous phosphate buffer (pH 7.5, 0.1M))
Hydroxyzine	2.6	4.7	1.80	2.60	4:96 v/v acetonitrile-buffer (pH 4, 0.1M)

[a] k1 and k2 are the retention factors of the first and second eluting enantiomers.

One exception is primaquine, which does not have either a carbonyl or an aryl function attached to chiral center, but undergoes separation on the avidin phase. The chiral recognition site or chiral recognition mechanism of primaquine may be different than other drugs. Francotte et al.[17] have reviewed single site/multiple mechanisms or multiple sites/multiple mechanisms of a chiral recognition event.

The stability of the avidin protein column in our laboratory was excellent. During the most recent six months period, retention times, separation factors, tailing factors, and resolution of enantiomers did not change significantly.

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CONCLUSION

The avidin column is sufficiently enantioselective and retentive to separate a large number of racemic pharmaceuticals. The pH, organic modifier, buffer strength, and anions of the mobile phase can be altered to manipulate retention and chiral recognition. The separation conditions of the selected chiral pharmaceuticals were optimized. The long term performance of the column was also monitored and found to be excellent. Analysis of the structures of the resolved analytes on the avidin column revealed the presence of either aryl or carbonyl functions directly attached to the chiral centers. The presence of either function may facilitate chiral recognition.

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