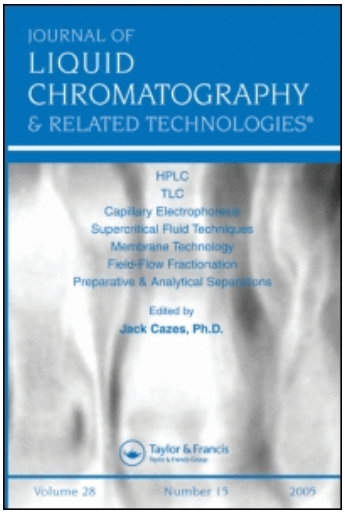

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Pre-Treatment of Chiral α-AGP Column with Triethylamine Significantly Improves the Detection Sensitivity of an Enantiomeric Leukotriene Antagonist

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PRE-TREATMENT OF CHIRAL α-AGP COLUMN WITH TRIETHYLAMINE SIGNIFICANTLY IMPROVES THE DETECTION SENSITIVITY OF AN ENANTIOMERIC LEUKOTRIENE ANTAGONIST

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ABSTRACT

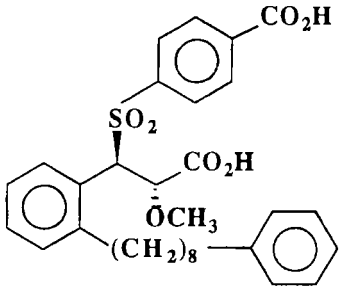
The peptidoleukotriene antagonist, SK&F 107310 and its optical antipode, can be effectively separated on a chiral α-AGP column. Pre-treatment of the chiral α-AGP column with 0.1% v/v triethylamine in the mobile phase significantly improves the peak shape and detection sensitivity of the undesired enantiomer. Chromatography with the modifier in the mobile phase resulted in elution of the drug substance without retention at the solvent front. Without column pre-treatment, only a broad tailing peak was obtained for enantiomer 2, making low detection of it unattainable.

INTRODUCTION

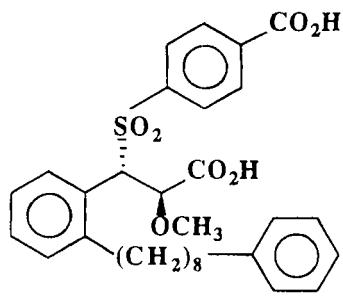
Leukotriene D_4, (LTD_4), formed from the metabolism of arachidonic acid by 5'-lipoxygenase, is considered an important receptor-mediator of human

bronchial asthma (1). Considerable efforts are ongoing in the pharmaceutical industry in pursuing specific leukotriene LTD_4 receptor antagonists as potential therapeutics for the treatment of asthma (2).

(R-(R*,S*))-ß-(4(carboxyphenyl)sulfonyl)-α-methoxy-2-(8-phenyloctyl)-benzenepropanoic acid (SK&F 107310) (**1**) is a potent and selective LTD_4 antagonist (3). The molecule has two chiral centers and therefore, four possible optical isomers. In order to evaluate the pharmacological profile of the single enantiomer **1**, we required an enantiospecific HPLC assay method to determine its chiral purity. Although **1** is structurally related to SK&F 106203 (4), our attempts to effect similar enantiomeric separation on all cellulosic columns were not successful. In this communication, we describe an enantiospecific HPLC method for the direct separation of SK&F 107310 (**1**) and its enantiomer **2** on a chiral α-AGP column, and report on a simple column pre-treatment technique that significantly improves the peak shape and, consequently, detection sensitivity of the enantiomer **2**. Without the column pre-treatment, only a broad triangular peak was obtained for the enantiomer **2**, making low detection of it unattainable.



(1)



(2)

MATERIALS AND METHODS

The liquid chromatograph system consisted of a Beckman Gold HPLC pump connected to an HP 1050 series autosampler and variable wavelength UV detector. A 4 x 100 mm second generation Chiral α-AGP column (Chrom Tech, obtained through Advanced Separation Technologies, Whippany, NJ) was used. Data was acquired and processed using a WATERS 860 Networking Computer System. All experiments were performed at ambient temperature.

Chemicals

Ethanol (200 Proof dehydrated alcohol, USP Punctilious grade) was obtained from Quantum Chemical Corporation (Newark, N. J.). HPLC water was obtained from J. T. Baker (Phillipsburg, N. J.) and anhydrous HPLC grade potassium dihydrogen phosphate was obtained from Fisher Scientific (Fair Lawn, N.J.). Triethylamine (99+% purity) was obtained from Aldrich (Milwaukee, WI).

SK&F 107310 (**1**) and the free diacid of its optical antipode (**2**) were synthesized in-house in the Department of Medicinal Chemistry. Both compounds were fully characterized by NMR, IR, MS, rotational measurements, elemental analysis and impurity profile by HPLC.

Column Pre-treatment

The mobile phase for the pre-treatment of the chiral α-AGP column consisted of a mixture of 40:60:0.1-ethanol: 20 mM potassium dihydrogen

phosphate buffer (pH 4.6 with no pH adjustment): triethylamine. The flow rate was 0.5 mL/min. The column was washed with this mobile phase for about 40 minutes, and then equilibrated with the assay mobile phase (see below) until a stable base line and constant retention time of **1** was obtained.

Chiral HPLC Assay

The mobile phase for the chiral HPLC assay consisted of a mixture of 40:60 ethanol: 20 mM potassium dihydrogen phosphate buffer (pH 4.6 with no pH adjustment) for Figures 1 and 2. This was changed to 37:63-ethanol: 20 mM potassium dihydrogen phosphate buffer (pH 4.6 with no pH adjustment) for Figure 3. This 37:63 ratio of ethanol:20 mM potassium dihydrogen

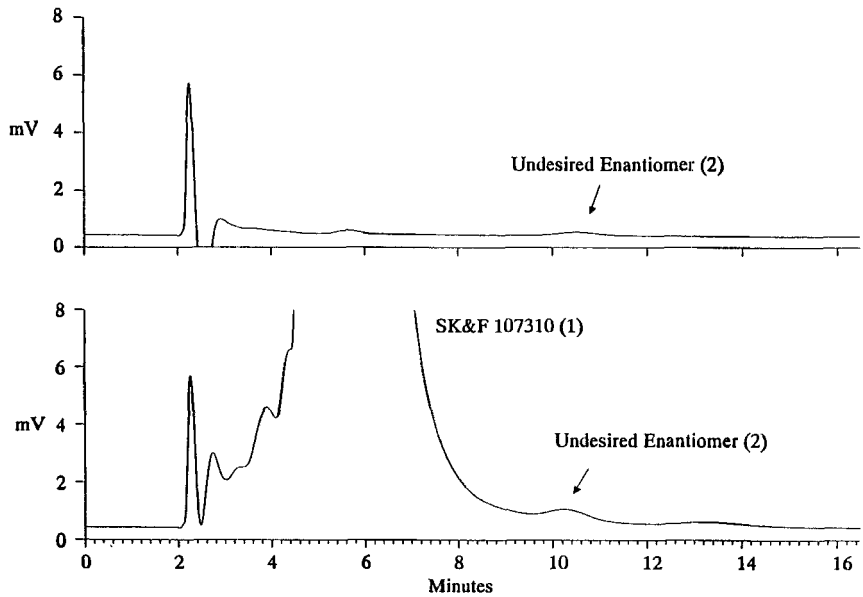


FIGURE 1. Chiral HPLC chromatogram on a brand new untreated Chiral α-AGP column. Top trace: 0.002 mg/ml **2**; Bottom trace: 0.002 mg/ml **2** spiked into 0.53 mg/ml **1** [0.4% w/w spike].

phosphate was also used for the buffer system at different pH (see Table 1), and with the addition of 1.2 mM to 12 mM of N, N-dioctylamine. The flow rate was 0.5 mL/min. UV detection was performed at 215 nm. The injection size was 25 μL unless otherwise indicated in the figure legend. For sample concentration, see figure legends.

Post-assay column care

The dihydrogen phosphate buffer in the chiral α-AGP column was purged with a mobile phase mixture consisting of 37:63-ethanol:water for about 40 minutes. The flow rate was 0.5 mL/min. The column was then stored in a refrigerator.

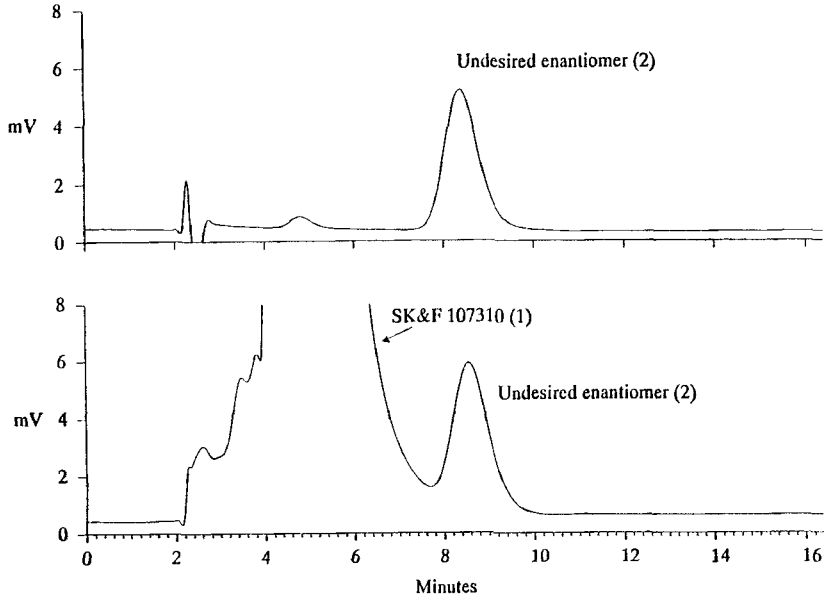


FIGURE 2. Chiral HPLC chromatogram on a pre-treated Chiral α-AGP column of identical sample solution as in Figure 2. Top trace: 0.002 mg/ml **2**; Bottom trace: 0.002 mg/ml **2** spiked into 0.53 mg/ml **1** [0.4% w/w spike].

RESULTS AND DISCUSSION

Protein-based chiral stationary phases have been found to be useful for the chromatographic resolution of a wide range of enantiomeric compounds (5). Good separation of the two optical antipodes **1** and **2** was also obtained with a second generation α-AGP column. However, very broad peak shape for the enantiomer **2** was obtained such that our required low detection of it at 0.5% w/w or lower in SK&F 107310 drug substance was not possible. The very broad peak shape for **2** was not a result of over-loading. At low concentration, the peak shape for **2** was also very broad that only an inflection of base line was observed (see Figure 1). Attempts at various pH adjustments of the mobile phase did not improve the chromatography.

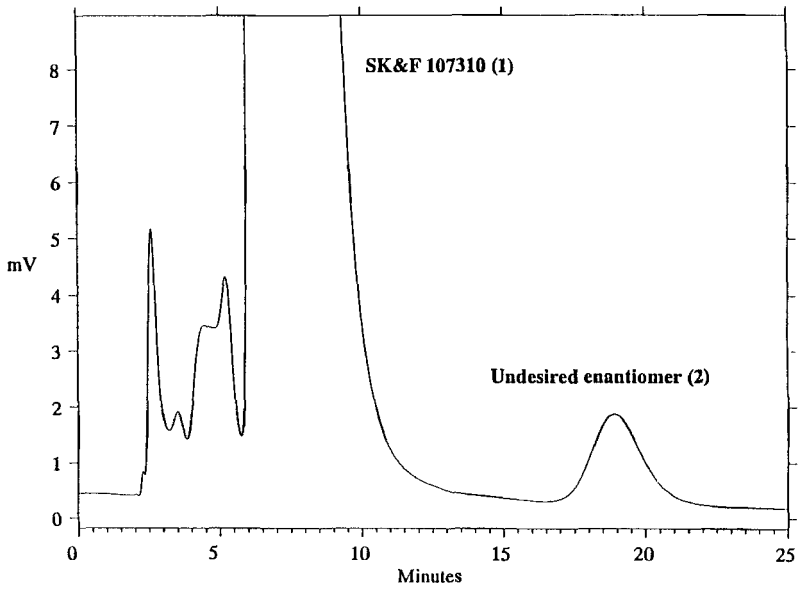


FIGURE 3. Optimized Chiral HPLC chromatogram on a pre-treated Chiral α-AGP column. Spiked at 0.4% w/w level : 0.002 mg/mL **2** spiked into 0.53 mg/mL **1**.

Since α_1-acid glycoprotein (orosomucoid) is known to contain large amounts of neuraminic acid that are located at the very end of the outer chain moiety of the sugar chains (6), we speculated that the broad tailing we observed for **2** may be a result of either residual column silanol effect or perhaps non-specific interaction between the analyte and the readily accessible neuraminic acid of the α_1-acid glycoprotein. We were further encouraged by the report that, although the carbohydrate chain is required for chiral recognition, neuraminic acids of ovomucoid in ovomucoid columns participate in a non-specific retention of acidic residues without affecting chiral resolution of, for example, ketoprofen (7). Accordingly, we attempted modification of the chiral α-AGP column by initially passing through the column for about 40 minutes an eluent that contained an additional 0.1% by volume of triethylamine. After due equilibration with the assay mobile phase (which does not contain triethylamine), the column was then used for analysis. Figure 1 shows the chromatography of a solution of the undesired enantiomer **2** at a concentration that would be equivalent to 0.4% w/w of **2** in **1** (bottom trace), and a 0.4% w/w spike of **2** (same concentration) in **1** (top trace) on an untreated chiral α-AGP column. Figure 2 shows the identical solutions injected after the aforementioned pre-treatment of the same column and analysis after due equilibration. As shown in Figures 1 and 2, the column pre-treatment with triethylamine significantly sharpens the peak shape and, consequently, lowers the detection limit of the undesired enantiomer **2**. The separation can be further optimized by reducing the amount of ethanol in the mobile phase from 40 to 37 %. This is shown in Figure 3. The tailing factor for the enantiomer **2** in this chromatogram is 1.2. Chromatography of the racemic mixture with a mobile phase containing 0.1% v/v triethylamine resulted in elution of the racemic mixture without retention at the solvent front. Similar addition of N,N-dimethyloctylamine in the

range 1.2 mM to 12 mM to the mobile phase (8), also resulted in the elution of **1** and **2** without retention at the solvent front.

We have repeated the same study with both a brand new chiral α-AGP column ourselves, and independently with a separate chiral α-AGP column by another analyst using radiolabelled **1**, and verified the technique to be indeed reproducible. The chiral α-AGP column, once pre-treated, can be stored in a refrigerator for months, and then reused without additional treatment. Indeed, we have validated the method on a pre-treated column that has been used for many prior assays and then stored in a refrigerator for months. The method is precise (% RSD 0.04 for **1** and 0.9% for **2** for twelve replicates), and is linear from 0.1 to 4 % w/w of the undesired enantiomer **2** in **1**. The detection limit is 0.1% w/w of the undesired enantiomer **2** in **1**.

The influence of eluent pH on retention and enantioselectiviety of **1** and **2** is shown in Table 1. As with other acidic solutes on untreated α-AGP column (8), the retentions of both **1** and **2** increase with decreasing pH. Changes in eluent pH also significantly affect enantioseparation and peak shape. Optimum retention, peak shape and separation were obtained at pH 4.6. At pH 3.0, although well separated from each other, **2** had an unacceptable retention time of 94 minute and a peak width of about 28 minutes. At pH 6.0, both **1** and **2** eluted without retention at the solvent front. Less than base-line separation was obatined at pH 5.0. Since the pH of the buffer solution containing 0.1% v/v of ethylamine, and 12 mM of N,N-dimethyloctylamine are 6.7 and 7.2, respectively, the elutions of **1** and **2** without retention at the solvent front are therefore most likely, a result of pH effect.

TABLE 1.

INFLUENCE OF ELUENT[a] pH ON RETENTION AND ENANTIOSELECTIVITY OF ENANTIOMERS 1 AND 2 ON PRE-TREATED α-AGP COLUMN

	pH 3.0[b]				pH 4.0				pH 4.4			
Compound	**k'**	**N**	**R_S[c]**	**Tailing**	**k'**	**N**	**R_S**	**Tailing**	**k'**	**N**	**R_S**	**Tailing**
SK&F 107310 (1)	19.8	735		1.5	11.1	867		1.5	3.9	587		1.5
Enantiomer (2)	45.9	329	3.9	1.8	22.0	536	3.9	1.4	6.9	398	2.6	1.4

	pH 5.0			
Compound	**k'**	**N**	**R_S**	**Tailing**
SK&F 107310 (1)	0.8	579		-
Enantiomer (2)	1.1	344	0.7	-

a 20mM potassium dihydrogen phosphate buffer (see Table for pH)-ethanol (63:37 v/v) was used as the eluent. A racemic mixture of 1 and 2 at a concentration of about 0.1 mg/mL was used. The injection size was 5 µL.

b Buffer pH adjusted with either phosphoric acid or sodium hydroxide.

c Resolution factor = $\frac{2 \times [\text{difference of retention times of (+) and (-) isomers}]}{[\text{Bandwidths of the two peaks}]}$

The precise mechanism leading to the sharpening of the peak shape and, consequently, improvements in detection sensitivity that we have observed in the present work, and whether this pre-treatment technique led to a "unique" column, is not clear. Although some applications for acidic compounds, e.g., fenoprofen and naproxen (8), ibuprofen (9, 10) and phenylbutyric acids (10) have been reported, chiral α-AGP column primarily has been used to resolve basic or cationic enantiomers (11-15). The separation factor for enantiomers of ibuprofen on chiral α-AGP column has been reported to increase with increasing concentration of (-) tetrolidine (16), and dimethyloctylamine (8), indicating the distribution of solutes as ion-pairs on the stationary phase. The addition of 1.2 mM dimethyloctylamine to the mobile phase has also been reported to improve the separation and peak shape of ibuprofen enantiomers on chiral α-AGP column (9). Other optimization of detection sensitivity on chiral α-AGP column has included gradient elution with increasing acetonitrile content in the mobile phase, as well as by pH gradient (17). Further work to determine the generality, as well as to probe the mechanistic aspect of our pre-treatment technique, is underway, and the results will be reported in a future communication.

CONCLUSIONS

SK&F 107310 and its enantiomer can be effectively separated on a chiral α-AGP column. Pre-treatment of the chiral α-AGP with triethylamine significantly improves the peak shape and detection sensitivity of the undesired enantiomer. Without column pre-treatment, only a broad tailing peak was obtained for the undesired enantiomer, making low detection of it unattainable.

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