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Short Communication

Chiral LC of a cholesterol-lowering drug using serum albumin mobile phases

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Introduction

Analytical techniques based upon natural substances are widely used to determine the enantiomeric purity of drugs. In particular, chiral LC has evolved at a rapid pace partly due to the development of packing materials to which proteins and enzymes, such as α_1-acid glycoprotein, ovomucoid, cellobiohydrolase and serum albumin, have been bound [1–5]. LC columns containing other natural products such as cyclodextrins, cellulose and amylose have also been broadly applicable [6–8]. In contrast, LC mobile phases containing proteins as chiral eluents, such as albumin and α_1-acid glycoprotein, have been shown to be analytically useful for the resolution of the enantiomers of only a few compounds [9–11]. Analytical chiral column methods are preferred due to greater sensitivity, higher possible column efficiency and comparatively easier methods development. However, the study of chiral eluents is useful for the development of possible applications by capillary electrophoresis (CE) [12, 13], in devising protein binding studies [13], and in cases where resolution is not possible using available chiral LC columns.

In this study, serum albumin was used as the chiral eluent in combination with achiral LC columns to resolve the enantiomers of an HMG CoA reductase inhibitor and cholesterol-lowering drug, SQ-33600 (Fig. 1). All of the commercially available chiral LC columns tried, including the ones based upon bovine serum albumin and human serum albumin, failed to resolve the enantiomer and only partial resolution was obtained when CE with albumin as chiral selector was tried. Albumin, a globular, transport protein stereoselectively binds a wide variety of substrates, particularly carboxylic acids [14]. Since SQ-33600 did not elute from a bovine serum albumin HPLC column at low pH (3.0), showed no chiral resolution at a higher pH (7.0) or upon addition of various modifiers, bovine serum albumin was, instead, added to the mobile phase in combination with a demethylsilane column. Chiral recognition was observed and a number of parameters were varied to optimize the separation.

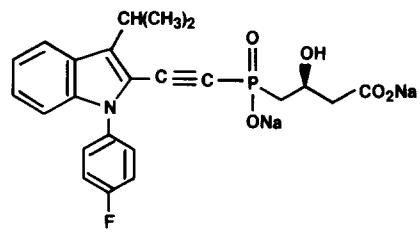

Figure 1
Structure of SQ-33600.

Experimental

Chemicals

SQ-33600 (*S*-4-[[[1-(4-fluorophenyl)-3-(1-methylethyl)-1H-indol-2-yl] ethynyl]hydroxy-

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phosphinyl]-3-hydroxybutanoic acid, disodium salt) and its enantiomer were obtained from the Bristol–Myers Squibb Pharmaceutical Research Institute. The various albumins, which were classified as essentially fatty acid free, were obtained from the Sigma Chemical Co. (St Louis, MO, USA). Potassium phosphate monobasic and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Distilled, deionized water was used throughout.

Chromatographic procedures

The mobile phases were prepared by adding potassium phosphate monobasic aqueous buffer (typically 0.05 M) and acetonitrile to a weighed quantity of albumin. The pH was adjusted by titrating with *o*-phosphoric acid. Dissolution of the albumin occurred by mixing, swirling and inverting the container. The mobile phase, which was clear, was filtered through a 0.45-μm sintered glass funnel and sonicated for about 10 min to remove air bubbles. The mobile phase was kept covered and was stored at 4°C (stable for 3 weeks) when not in use to retard bacterial growth. The Chromegabond, Nucleosil and Lichrosorb analytical columns were obtained from ES industries (Marlton, NJ, USA). All Chromegabond columns (C-1–C-18), the Lichrosorb diol, Nucleosil cyano and the Nucleosil diol were 25 cm × 4.6 mm with a 5-μm particle size and a 60 Å pore size. The column temperature was controlled using a thermostatted column heater from Jones Chromatography (Boulder, CO, USA).

The LC system consisted of a Beckman Model 110B single piston pump (Beckman, Somerset, NJ, USA), a Perkin–Elmer Model ISS-100 autoinjector with a 20-μl sample loop (Perkin–Elmer-ABI, Elmwood Park, NJ, USA) and an ABI 783 absorption detector set to 300 nm. Data acquisition was accomplished using a VG Multichrom system (VG/Fisons, Danvers, MA, USA). Equilibration of the LC system takes about 30 min at a flowrate of 0.5 ml min^{-1}. The optimum sample concentration is 0.2 mg ml^{-1} in mobile phase.

Results and Discussion

Initial separation

Resolution of the enantiomer of SQ-33600 was not attained using α_1-acid glycoprotein, bovine serum albumin, human serum albumin, ovomucoid, β-cyclodextrin, γ-cyclodextrin or cellulose LC columns (Chiralcel OB, OC, OD and OJ) nor by using CE with various cyclodextrins as chiral selectors. The chiral LC columns were investigated under a variety of mobile phase conditions. Although partial resolution was obtained using CE and albumin as additive, time constraints did not permit possible further development. When bovine serum albumin, dissolved in aqueous phosphate buffer, was used as the chiral eluent in combination with a Chromegabond dimethyl (C-2) column, satisfactory chiral resolution was

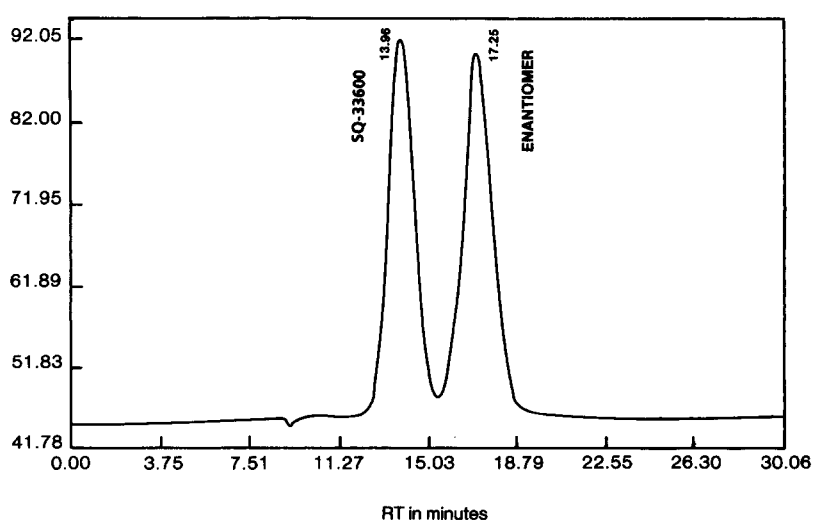

Figure 2
Chromatogram of SQ-33600 and its enantiomer (a racemic mixture) using bovine serum albumin as the chiral discriminator; conditions: 7.0 g bovine serum albumin/l, Chromegabond C-2 column, 25 cm × 4.6 mm, 5-μm particle size, 0.04 M potassium phosphate buffer, pH 6.3, 0.2 ml min^{-1}, UV absorption detection at 300 nm, sample concentration 0.2 mg ml^{-1} total in mobile phase, time vs response.

observed (Fig. 2). The limit of detection of the unwanted enantiomer was not acceptable (about 0.2% w/w) partly due to the background noise caused by the excessive quantity of bovine serum albumin required (7 g l^{-1}) to elute SQ-33600 from the column and an undesirable elution order. An investigation of several parameters with the objective of improving the detection limit was performed.

Variation of parameters

Albumins derived from the various species differ in structure and can have different binding affinities for the same drug compound. The effect of varying the species of albumin on chromatographic selectivity has been noted previously [10]. Several types of albumins were tried in an attempt to improve selectivity as well as resolution (Table 1). Optimization and investigation of the procedure proceeded to a greater extent using horse albumin, since the reversed elution order was preferred, less material (relative to bovine albumin) was required to obtain resolution and none of the potential impurities coeluted with the opposite enantiomer.

A number of LC columns were tested and there was a significant effect on chiral resolution when changing column types. Baseline resolution was observed using Chromegabond C-1 and C-2 columns. Only partial resolution was obtained when using some diol and cyano columns (Lichrosorb and Nucleosil brands). Elution of the isomers was not observed using the more hydrophobic Chromegabond hydrocarbon-containing columns (C-3 or large carbon number). It is likely that the more hydrophobic stationary phases adsorb the albumin thus causing excessive retention of the analyte. The adsorption of human serum albumin to C-8 and phenyl stationary phases but not to a more polar diol column has been shown to occur by using the technique of frontal analysis [9].

The acetonitrile content was varied when using horse albumin as the chiral selector (Table 2). Resolution was obtained only when

Table 1
Effect of the type of albumin upon elution order and resolution (R_s)*

Type of albumin	Elution order	Maximum R_s
Rabbit	No resolution	No resolution
Horse	R, S	2.1
Cow	S, R	1.4
Sheep	S, R	1.3
Pig	R, S	1.6

*The chromatographic conditions were varied and optimized to attain the best resolution for each species. A Chromegabond C-2 column was used.

Table 2
Effect of acetonitrile on resolution (R_s) and retention times*

% Acetonitrile	Enantiomer retention time (min)	SQ-33600 retention time (min)	R_s
0	14.3	14.3	No resolution
5	12.1	17.1	1.2
10	13.1	19.1	2.1
15	24.4	33.4	1.4

*Conditions: 1 g l^{-1} horse albumin, 0.05 M potassium phosphate buffer pH 5.5, Chromegabond C-2 column, 25 cm × 4.6 mm, 5-μm particle size, 0.5 ml min^{-1}, 40°C column temperature, 300 nm UV absorption detection.

Table 3
Effect of pH on resolution (R_s) and retention times*

pH	Enantiomer retention time (min)	SQ-33600 retention time (min)	R_s
6.5	9.6	12.4	1.1
6.0	9.5	13.8	1.4
5.5	13.3	20.5	2.1

*Conditions: 1 g l^{-1} horse albumin, 10% acetonitrile, 90% 0.5 M potassium phosphate monobasic, pH adjusted by titrating with *o*-phosphoric acid, Chromegabond C-2 column, 25 cm × 4.6 mm, 5-μm particle size, 0.5 ml min^{-1}, 40°C column temperature, 300 nm UV absorption detection.

acetonitrile was added. A maximum in resolution occurred at 10% acetonitrile and the retention times became longer as the acetonitrile was increased from 5 to 15%. It is well known that various conformers of proteins can occur when the solvent properties are altered. In particular, C1–C2 cosolvents have been described as "penetrating" and cause swelling or expansion of proteins [15]. Changes in the tertiary structure of albumin undoubtedly can alter the nature of the binding site and the protein's binding affinity for a particular drug and, therefore, effect chiral resolution as well as retention times when used as a chiral additive in the mobile phase.

The data in Table 3 shows that lowering the pH improves resolution and increases retention times. The conformational state of human serum albumin has been shown to undergo a transition with a variation in pH over the range of pH 5.0–9.0 [16]. The change in the conformational state and surface charge of albumin as occurs with pH variation can alter the binding constant for certain drugs, which would cause a change in the chromatographic properties with pH [16]. However, since the pK_as of the carboxylic and phosphinic acid functional groups have been estimated by a solubility profile study to be 5.1 and 2.0, respectively, a change in the form of the drug as the pH is lowered (protonation of the carboxylic group) may also be effecting the drug/protein interaction as well as the chromatographic properties (personal communication, Dr A. Serajuddin, Bristol–Myers Squibb Pharmaceutical Research Institute, June 1994).

Due to the large quantity of horse albumin used, there is no enhancement in resolution by using a concentration of albumin greater than 1 g l^{-1}. Although the use of higher concentrations results in sharper peaks and reduced retention times, the use of 1 g l^{-1} is preferred over 2 g l^{-1} because there is less background absorption due to the mobile phase, and thus, less noise and greater sensitivity. When concentrations of 0.01–0.08 g l^{-1} were tried, baseline resolution was obtained but the peaks were asymmetrical (with frontal tailing) and broader. For example, although the resolution factor was 2.0 using a mobile phase with an albumin concentration of 0.1 g l^{-1}, the asymmetry factors were 0.2 and 0.3 for the enantiomer and SQ-33600, respectively. A concentration of 1 g l^{-1} was selected because of the resultant improved peak symmetry (typically, asymmetry factors of 0.5 and 0.8 for the enantiomer and SQ-33600, respectively) and adequate resolution (resolution factors of 1.3 or greater).

The column temperature was increased to 40°C to sharpen the peaks. There was no significant effect on resolution observed by raising the column temperature. When the column temperature was raised to 45°C, a decrease in resolution was observed and the baseline became noisy, possibly due to denaturing of the protein. The optimized separation is shown in Fig. 3 with enantiomer added at 0.05% w/w.

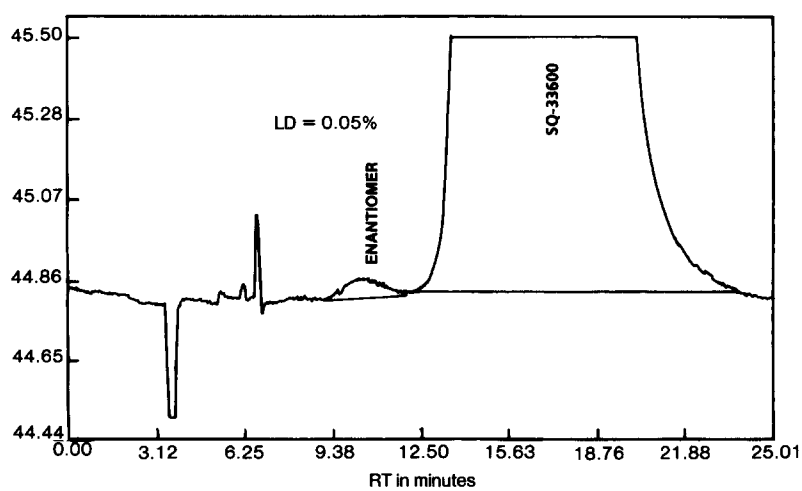

Figure 3
Chromatogram of SQ-33600 (0.2 mg ml^{-1} in mobile phase) with 0.05% w/w opposite enantiomer added using horse albumin as the chiral discriminator under the optimized conditions; conditions: 1 g l^{-1} horse albumin, 10% acetonitrile, 0.05 M potassium phosphate buffer pH 5.8, 0.5 ml min^{-1}, 40°C, Chromegabond C-2 column, 25 cm × 4.6 mm, 5-μm particle size, UV absorption detection at 300 nm, time vs response.

Assay validation and application

The enantiomeric assay was validated using the optimized conditions described in the caption of Fig. 3. Linearity of the response of SQ-33600 was excellent in the 0.035–0.40 mg ml^{-1} concentration range (coefficient of correlation of 0.999) and was not tested at higher concentrations. The linearity of the enantiomer response was excellent in the 0.1–4% concentration range (w/w relative to SQ-33600 at 0.2 mg ml^{-1}; coefficient of 0.998). Reproducibility of SQ-33600 response was satisfactory with a RSD of 1.2% for six determinations. Recovery of the enantiomer added to SQ-33600 at various concentrations (0.4–4.0% w/w) was satisfactory and ranged from 98 to 105%. The limit of detection was about 0.05% w/w, estimated using a statistical linearity method. Ruggedness was acceptable since there was no significant column-to-column variation. However, columns needed to be replaced after about 15–20 working days (about 8–10 h d^{-1} of use). As the column aged due to use, a loss in resolution and broadening of the peaks was observed. The columns were rinsed with water and stored overnight in 10% acetonitrile/water to prevent the precipitation of mobile phase components.

The assay was essential for monitoring the enantiomer content of bulk drug substance during the optimization of the synthetic process and for the determiantion of enantiomer in the capsule dosage form and bulk drug substance during stability studies. Racemization of SQ-33600 was not observed under the various conditions of high temperature and humidity. In several instances, the enantiomer content of some developmental bulk batches were reconfirmed by reanalyzing the samples using bovine or pig albumin as chiral additive. This was done to utilize the different selectivities obtained by the various albumins to reduce the probability of an impurity coeluting with the unwanted enantiomer and to support the optimized method's results.

Conclusion

A direct LC method using horse albumin as a chiral mobile phase additive in combination with a dimethylsilane column was developed, validated and utilized to determine the enantiomer content of bulk and formulated SQ-33600, a cholesterol-lowering drug. Although the use of chiral mobile phase additives in LC is more complex and less sensitive than chiral column methods, the reported assay was required due to the failure to obtain useful chiral resolution by using various commercially available columns as well as by chiral CE.

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