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# Separation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor drug substance diastereomers, and their analogues on β-cyclodextrin stationary phase

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**Abstract**

β-Cyclodextrin stationary phases are extremely useful in the separation of complex diastereomeric mixtures under normal-phase chromatographic conditions. The retention behavior of the 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A reductase inhibitor drug substances (I–IV) is influenced by the size and chain length of the polar alcohol modifier. Retention time changes caused by different alcohol modifiers can be explained by hydrogen bonding and steric effects involving the stationary phase, the analyte and the alcohol modifier.

## 1. Introduction

The utility of cyclodextrin stationary phases as chromatographic chiral selectors in the separation of enantiomers is well established [1–6]. In the reversed-phase mode of chromatography, the mechanism of chiral separation afforded by the cyclodextrin stationary phase is believed to involve the formation of an inclusion complex between the chiral analyte and the hydrophobic cavity of the cyclodextrin molecule [6,7]. This phenomenon of cavity inclusion complexation by cyclodextrins is not limited to its application as chiral separation agent. Recently, this property of cyclodextrins has become increasingly popular in enhancing the aqueous solubility, bioavailability, and stability of pharmaceutical drug substances [8,9]. In the reversed-phase mode of chromatography, the hydroxyl groups on the outer side of the cyclodextrin molecules play an important role by providing hydrogen-bonding controlled stabilization of the inclusion complex. In the normal-phase mode of chromatography employing a non-polar hydrocarbon solvent, the hydrophobic cavity of the cyclodextrin molecules is occupied by the non-polar solvent molecules, and this cyclodextrin–hydrocarbon complex offers a unique environment consisting of primary and secondary hydroxyl groups and ether oxygen atoms. β-Cyclodextrin has a C-7 symmetry axis and 14 hydroxyl groups situated about the mouth of the cavity. Seven of these are at the C-2 carbon of the glucose and orient in the clockwise direction, while those at C-3 position are anticlockwise. The opposite rim of the cavity has seven primary hydroxyl groups. The potential of

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three types of functionalities in providing interactions with compounds possessing hydrogen bonding sites for unique chromatographic selectivities appears very attractive.

We have studied the utility of cyclodextrin stationary phases in the normal-phase mode of chromatography in the separation of a complex mixture of diastereomers of our HMG-coenzyme A (CoA) reductase inhibitor drug substance (RG 12561) and related compounds. Preclinical as well as clinical studies show that RG 12561, [4α,6β(*E*)]-(±)-6-[2-[2-(4-fluoro-3-methylphenyl)-4,4,6,6-tetramethyl-1-cyclohexen-1-yl)ethynyl]tetrahydro-4-hydroxy-2H-pyran-2-one (I, Fig. 1) is an effective and potent HMG-CoA reductase inhibitor and hypocholesterolemic agent. RG 12561 possesses the β-hydroxy-4-lactoneethylenic moiety deemed necessary for the activity and present in the naturally occurring substances of this class. The drug substance RG 12561 is prodrug for the corresponding open-chain dihydroxy acid (IV, Fig. 1). Synthesis of RG 12561 involves stereospecific reduction of the hydroxyketo ester X or XI (Fig. 1) to produce the corresponding *syn*-dihydroxy esters VI and VIII, respectively, followed by de-esterification to the free acid (IV, Fig. 1) and lactonization. Under non-ideal conditions of reduction of the above two esters, the corresponding anti-dihydroxy esters (VII, IX, Fig. 1) could be formed. Also, the lactones I and II could be labile to hydrolytic and transesterification conditions and produce diastereomeric free acids IV and V, respectively, or the corresponding esters VI and VII, as the case may be. The formation of the product III is also observed under dehydration conditions. Development of RG 12561 required a chromatographic method which was capable of providing separation of the above diastereomeric compounds and III in one chromatographic run.

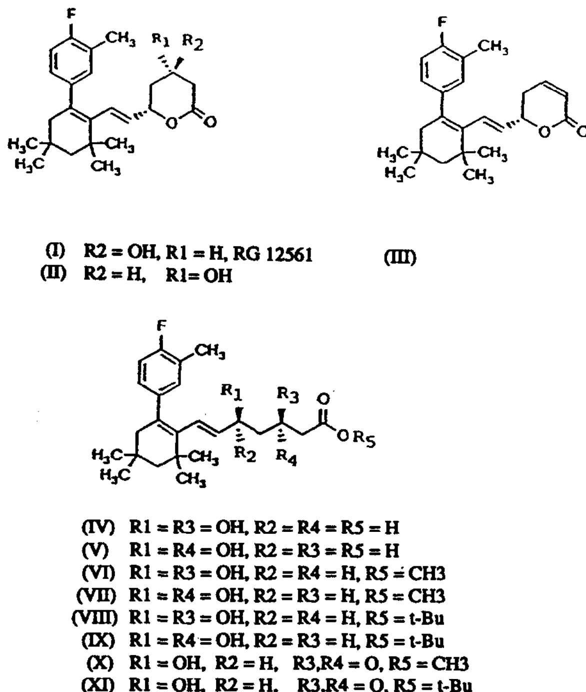


Fig. 1. Structures of RG 12561, its stereoisomers and related compounds. t = *tert*.

## 2. Experimental

### 2.1. Chemicals

The drug substance RG 12561 and related compounds II–IX were synthesized by the Process Research Department at Rhône-Poulenc Rorer Central Research, Collegeville, PA, USA. HPLC-grade hexane, methylene chloride, isopropanol and trifluoroacetic acid and reagent-grade *n*-propanol, *n*-butanol, *tert.*-butanol and *tert.*-amyl alcohol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). 2-Butanol was purchased from EM Science (Gibbstown, NJ, USA). 2-Pentanol was purchased from Aldrich (Milwaukee, WI, USA). Dioxane, analyzed reagent grade, was obtained from J.T. Baker (Phillipsburg, NJ, USA). Quantum Chemical (Tuscola, IL, USA) supplied ethanol, 200-proof dehydrated alcohol, USP.

### 2.2. Equipment

An HPLC pump (Spectra-Physics 8800 solvent-delivery system), an autosampler (Spectra-Physics 8800 Autoinjector) and a variable-wavelength UV detector (Spectra-Physics Forward optical scanning detector) were used. Data ac-

quisition was done with an integrator/computer (IBM PS/2 Model 70 equipped with Spectra Focus software). Other equivalent equipment, along with Waters Chromatography ExpertEase software, was also used.

### 2.3. Chromatographic conditions

The primary mobile phase consisted of hexane–ethanol (96:4, v/v; equivalent to 0.682 *M* ethanol) containing 0.1% (v/v) trifluoroacetic acid. Other mobile phases were prepared by mixing hexane and different alcohols at 0.682 *M* concentration and adding 0.1% (v/v) trifluoroacetic acid. The flow-rate was 2.0 ml/min. In a typical analysis, a 20-$\mu$l volume of the sample solution was injected into two serially linked Cyclobond I columns, each 250 × 4.6 mm I.D., 5 $\mu$m particle size (Advanced Separation Technologies, Whippany, NJ, USA). Detection was performed by a UV detector set at 245 nm. Retention characteristics measured as capacity factors ($k'$) were computed from the equation $k' = t_R - t_0/t_0$, where $t_R$ and $t_0$ are retention times of retained and unretained compounds, respectively. Methylene chloride was injected as a measure of the unretained compound.

### 2.4. Standard and sample preparations

Solutions of RG 12561 and related compounds were prepared in a diluent consisting of hexane–*tert.*-butanol (95:5, v/v). For the linearity study, a set of solutions of RG 12561 of concentrations 0.54, 0.79, 1.06, 1.25 and 1.53 mg/ml which range from approximately 50 to 150% of the target assay concentration of 1.0 mg/ml were prepared. To determine system precision of the method, a standard solution of 0.97 mg/ml was used. Method precision was determined using six solutions of concentrations ranging from 0.88 to 0.92 mg/ml. In order to study retention characteristics and the separation mechanism of four diastereomers on the Cyclobond I column using different polar alcohol modifiers in the mobile phase, a 1.0 mg/ml solution of RG 12561 containing approximately 0.1 mg/ml each of II (RG 12841), IV (RG 12839) and V (RG 14100) were prepared.

## 3. Results and discussion

Attempts to achieve the separation of compounds I–XI (Fig. 1), which include four diastereomeric pairs, under reversed-phase conditions of chromatography employing a variety of stationary phases met with difficulties due to partial or no separation of the above compounds. Use of two serially linked $\beta$-cyclodextrin columns in the normal-phase mode of chromatography employing a mobile phase consisting of hexane–ethanol containing 0.1% of trifluoroacetic acid produced the desired separation. A chromatogram showing the separation of related compounds, including four diastereomer pairs and compound III is depicted in Fig. 2. The capacity factors of these compounds under these

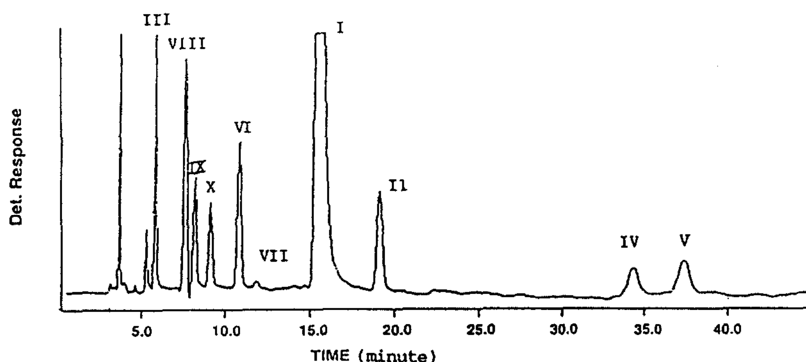


Fig. 2. HPLC Chromatogram of RG 12561 and related substances.

Table 1
Effect of alcohol modifiers on the retention times and capacity factors of RG 12561 and related compounds

| Alcohol | Compounds | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | RG 12561 (I) | | RG 12841 (II) | | RG 12839 (IV) | | RG 14100 (V) | |
| | $t_R$ | $k'$ | $t_R$ | $k'$ | $t_R$ | $k'$ | $t_R$ | $k'$ |
| *Primary alcohols* | | | | | | | | |
| $CH_3$–$CH_2$–OH | 16.39 | 4.6 | 20.28 | 5.9 | 35.54 | 11.1 | 38.96 | 12.3 |
| $CH_3$–$CH_2$–$CH_2$–OH | 15.05 | 4.0 | 18.53 | 5.2 | 30.46 | 9.2 | 32.95 | 10.0 |
| $CH_3$–$CH_2$–$CH_2$–$CH_2$–OH | 15.20 | 4.1 | 18.97 | 5.3 | 26.99 | 8.0 | 29.70 | 8.9 |
| $CH_3$–$CH_2$–$CH_2$–$CH_2$–$CH_2$–OH | 14.66 | 3.9 | 18.35 | 5.1 | 29.65 | 8.9 | 32.00 | 9.7 |
| *Secondary alcohols* | | | | | | | | |
| $CH_3$–CH(OH)–$CH_3$ | 16.94 | 4.6 | 21.15 | 6.0 | 29.36 | 8.8 | 32.53 | 9.8 |
| $CH_3$–$CH_2$–CH(OH)–$CH_3$ | 18.62 | 5.7 | 23.80 | 7.6 | 29.96 | 9.8 | 33.47 | 11.0 |
| $CH_3$–$CH_2$–$CH_2$–CH(OH)–$CH_3$ | 19.24 | 5.8 | 25.19 | 7.9 | 28.71 | 9.1 | 31.99 | 10.3 |
| *Tertiary alcohols* | | | | | | | | |
| $(CH_3)_3$–C–OH | 23.36 | 7.0 | 30.89 | 9.6 | 31.72 | 9.9 | 34.92 | 11.0 |
| $(CH_3)_2$–C(OH)–$CH_2$–$CH_3$ | 24.38 | 7.3 | 32.38 | 9.9 | 29.62 | 9.0 | 33.81 | 10.5 |

conditions of chromatography are listed in Table 1. Under the conditions described in this method, the two dihydroxy acids showed relatively large retention times. Validation studies were carried out with respect to RG 12561 (I). The method provided satisfactory validation parameters for quantitative use of the method. The validation data are summarized in Table 2. A linearity study was performed using five solutions of concentration ranging from 50 to 150% of the target concentration of 1.0 mg/ml. The correlation coefficient of the fitted line was 0.99999. System precision of six injections of a standard solution of 0.97 mg/ml concentration had an R.S.D. of 0.9%. Method precision determined for six different sample preparations ranging in concentration from 0.88 to 0.95 mg/ml showed an R.S.D. of 1.2%. Recovery studies were performed at nine levels of concentration covering the range of 50–150% of target assay concentration of 1.0 mg/ml. The mean recovery was 100.4% with an R.S.D. of 0.8%. The limit of detection of the method as determined by a signal-to-noise ratio of 3 was found to be 2 ng on column.

Compounds IV and V have three protic functional groups capable of hydrogen bonding. They are retained longer than the lactones. The retention of the anti-dihydroxy acid V is greater than the *syn*-diastereomer IV in all the mobile phase systems examined in this study. Intramolecular hydrogen bonding in the latter could possibly compete with the hydrogen-bonding interactions with the stationary phase. We

Table 2
Method validation data for RG 12561 (I)

| | |
|---|---|
| Linearity | $r$ ($n$ = 5): 0.99999 |
| Instrument precision | R.S.D. ($n$ = 6): 0.9% |
| Method precision | R.S.D. ($n$ = 6): 1.3% |
| Recovery | Mean assay ($n$ = 9): 100.4% |
| | R.S.D. ($n$ = 9): 0.8% |
| Limit of detection (RG 12561) | 2 ng on column (signal/noise ratio = 3) |

investigated the effect of chain length and steric bulk of the alcohol modifier on the retention times and capacity factors of the four key compounds, the lactones I and II, and their corresponding free acids IV and V. On increasing the chain length of the alcohol modifier from ethanol to *n*-butanol, the retention of the dihydroxy acids IV and V diminishes. Increase in the chain length is expected to increase the hydrophobic character of the hydrogen bonded complex including the protic groups of IV and V and the alcohol. The effect reverses with *n*-pentanol which provides retention greater than *n*-butanol for compounds IV and V. Possible inclusion of the long hydrophobic chain of *n*-pentanol in the cyclodextrin cavity and diminished efficiency of hydrogen bonding between the analyte and multiple *n*-pentanol molecules may be responsible for this reversal. The changes in retention times of the lactones I and II with the increasing alcohol chain length are relatively smaller. Among the homologous secondary and tertiary alcohols, the effect of increase of the carbon chain length of the alcohols on the retention times of IV and V is very small, but the lactones I and II show significant retention increases.

### *3.1. Effect of increase in the steric bulk of alcohols*

On going from the straight-chain alcohols to their branched-chain isomers, the behavior of the acids IV and V and the lactones I and II is reversed. The branching has little influence on the retention times of IV and V but the retention times of the lactones I and II show a marked increase in propanol, butanol and pentanol series. In I and II, the hydrogen bonding with the first bulkier alcohol molecule may diminish the possibility of further hydrogen bonding due to steric hindrances. On the other hand, the dihydroxy acids IV and V have a much more flexible open chain. The inhibition of multiple hydrogen bonding due to steric crowding is expected to be relatively smaller.

The use of dioxane as the polar modifier resulted in the large retention of all of the compounds. Hydrogen-bonded interaction between the analyte, protic modifiers and cyclodextrin hydroxyl groups play a key role in the retention behavior of the analyte. In the absence of protic functionality in the mobile phase with dioxane, a large retention on the hydroxylic stationary phase is expected.

## 4. Conclusions

$\beta$-Cyclodextrin provides an excellent stationary phase for the normal-phase chromatographic separation of diastereomers possessing hydrogen-bonding sites. The separation mechanism appears to be dominated by the hydrogen-bonded interactions of the analyte with the cyclodextrin and the alcohol modifiers.

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