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CHROMATOGRAPHIC SEPARATION OF THE DIASTEREOMERS OF A DIHYDROPYRIDINE-TYPE CALCIUM CHANNEL ANTAGONIST AS THE BIS-3,5-DINITROBENZOATES

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SUMMARY

The diastereomeric components of the calcium channel antagonist RS-93522-004 are separated as the bis-3,5-dinitrobenzoate ester by reversed-phase high-performance liquid chromatography. The diastereomer ratio determination is shown to be precise, accurate and sensitive, and is not affected by the reaction yield of the derivatization with 3,5-dinitrobenzoyl chloride. The four individual stereoisomers of RS-93522-004 were independently shown to have equal reactivity toward 3,5-dinitrobenzoyl chloride.

INTRODUCTION

2-[4-(2,3-Dihydroxypropoxy)phenyl]ethyl methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (1), is a dihydropyridine-type calcium channel antagonist. It is being developed by Syntex Research under the code number RS-93522-004 for treating congestive heart failure and possibly hypertension. RS-93522-004 is unique among known dihydropyridine calcium entry blockers insofar as it features two asymmetric carbons and therefore exists as a mixture of four stereoisomers. More precisely, they consist of two enantiomeric pairs of diastereomers. The two asymmetric carbons, one at the junction of the dihydropyridine and phenyl rings and the other at the secondary hydroxyl group, are separated by 11 atoms. Animal studies on each individual isomer have shown that the most active isomers are those which have the (*S*) configuration at the C-4 (dihydropyridine ring junction) carbon. These would be the (*S*,*R*) and (*S*,*S*) isomers. Since the drug is currently being studied as the racemic mixture, quantitation of the amount of each isomer would be informative from a clinical as well as a regulatory standpoint. As an alternative, one may choose to monitor the ratio of the diastereomer pairs.

The application of chiral high-performance liquid chromatography (HPLC) columns for diastereomer separation has been proposed[1] and several applications have appeared in the literature. Pirkle *et al.* have reported the diastereomeric separations of the α-naphthamide of a quinoline[2], 3,5-dinitrobenzoyl α-amino acids[3], bis-3,5-dinitrophenyl carbamates of alicyclic diols[4], as well as di-and tri-peptides[5].

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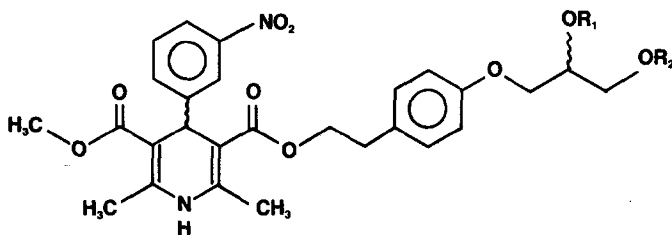
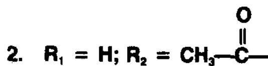
Extreme selectivity (resolution greater than 80) has also been observed for the diastereomers of 1,10-bis-(3,5-dinitrobenzoylleucine)decane by Pirkle and Pochapsky[6]. It is of interest to note, however, that recent reviews on this subject[7–20] do not mention the use of chiral HPLC columns for the resolution of diastereomers.

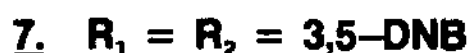
In a continuing program of analyzing the enantiomeric and diastereomeric purity of all new research compounds, we have successfully separated the diastereomers of an angiotensin-converting enzyme inhibitor using either a Pirkle-type column or an α_1-acid glycoprotein chiral column. In the present work, a number of commercially available chiral HPLC columns were investigated for their ability to separate the individual optical isomers or diastereomers of RS-93522-004. The columns investigated include: (1) Pirkle covalent phenyglycine; (2) Pirkle covalent naphthylalanine; (3) Cyclobond β-cyclodextrin; (4) Resolvosil bovine serum albumin; and (5) Enantiopak α_1-acid glycoprotein columns. The mobile phases employed were the ones typically suggested for chiral separation by either the column manufacturers or presented in the literature. In no case did the individual optical isomers or diastereomers of RS-93522-004 resolve on the columns tested.

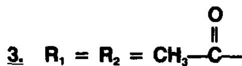
The remainder of this work focused on the separation of the diastereomer pairs of RS-93522-004. Preliminary analysis of RS-93522-004 using conventional reversed-phase and normal-phase HPLC with a variety of mobile phases gave no separation of the diastereomers, although in many attemps the compound eluted as a very broad peak, suggesting partial resolution of the diastereomers.

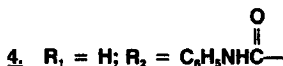
Since RS-93522-004 contains a diol functionality which could be easily derivatized, several different derivatives were prepared in an effort to achieve either indi-

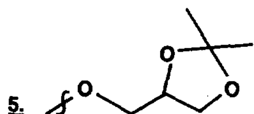












vidual optical isomer or diastereomer separation. RS-93522-004 was derivatized with: (i) acetic anhydride, producing both the monoacetate (2) and the diacetate (3); (ii) phenylisocyanate[21], producing only the monoderivative (4); (iii) 2,2-dimethoxypropane[22], producing the acetonide (5); and (iv) 3,5-dinitrobenzoyl chloride[23], producing both the mono- (6) and bis- (7) derivatives.

Each derivative was analyzed by conventional reversed-phase and normal-phase HPLC, as well as by using the above mentioned chiral HPLC columns. Only compound 7, the bis-3,5-dinitrobenzoate (bis-3,5-DNB) derivative, afforded separation of the diastereomer pairs. This was accomplished on a reversed-phase system using methanol–water as the mobile phase (Fig. 1). Again, the bis-3,5-DNB derivative did not separate into diastereomers on any of the chiral columns tested.

EXPERIMENTAL

Apparatus

The HPLC equipment consisted of a Spectra-Physics Model 8100 liquid chromatograph equipped with a Valco fixed-loop injector and a Hewlett-Packard 1040A photodiode array UV–VIS detector set at 254 nm. The detector output was monitored using a Spectra-Physics SP4000 recording integrator.

Materials

RS-93522-004 and its optically pure stereoisomers were synthesized by the Institute of Organic Chemistry, Syntex Research, Palo Alto, CA, U.S.A. 3,5-Dinitrobenzoyl chloride (98%+) and triethylamine (99%+) were obtained from Aldrich (Milwaukee, WI, U.S.A.). HPLC-grade methanol and dichloromethane were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Double distilled deionized water was used throughout the experiments.

Chiral HPLC columns

The chiral HPLC columns used were: (1) Pirkle Covalent D-Phenylglycine (Regis, Morton Grove, IL, U.S.A.); (2) Pirkle Covalent D-Naphthylalanine (Regis); (3) Cyclobond β-cyclodextrin (Astec, Whippany, NJ, U.S.A.); (4) Resolvosil bovine serum albumin (Machery Nagle, Düren, F.R.G.); and (5) Enantiopak α_1-acid glycoprotein (LKB, Bromma, Sweden).

Derivatization procedures

In a dry 75 × 12 mm I.D. test tube 44 mg (approximately 10-fold excess) of 3,5-dinitrobenzoyl chloride and 10 mg of RS-93522-004, or the individual stereoisomer, are dissolved in 3.0 ml of dichloromethane. A 26-μl volume of triethylamine (in 1.0 ml dichloromethane) is slowly added to the reaction mixture. The test tube is gently shaken intermittently over a 10–15-min period. A white precipitate appears after about 5 min. The reaction mixture is then evaporated to dryness and the product is dissolved in 5.0 ml of acetonitrile and slowly filtered through a C_{18} Sep-Pak (Waters, Milford, MA, U.S.A.). The reaction mixture is diluted to 25 ml with acetonitrile and further diluted to 100 ml with the HPLC mobile phase prior to HPLC analysis.

Chromatographic conditions

Determination of the diastereomer ratio was performed on an Alltech Spherisorb C_8, 5 μm, 250 × 4.6 mm I.D. HPLC column (Alltech Deerfield, IL, U.S.A.) using methanol–water (63:37, v/v) as the mobile phase. The column was maintained at 40°C. The mobile phase was delivered at 2.0 ml/min and the column eluent was monitored at 254 nm. The sample loading was typically 4 μg of the derivatization product with a 25-μl sample loop.

For the preparative HPLC isolation of the resolved diastereomers, an Alltech Ultrasphere C_8, 5 μm, 250 × 10 mm I.D. column maintained at 40°C was used. The mobile phase was methanol–water (65:35, v/v) and the flow-rate was 6.0 ml/min. The column eluent was monitored at 254 nm and the sample loading was 1.0 mg.

RESULTS AND DISCUSSION

Identification of diastereomers

Reversed-phase HPLC of the bis-3,5-DNB derivative (7) afforded the chromatogram shown in Fig. 1. The diastereomers of 7 separate into two peaks which elute at about 41 and 44 min. These will be referred to as peaks A and B, respectively. Peaks A and B were identified as diastereomers by isolation of the individual peaks using preparative HPLC, and characterization of each by spectroscopic analyses. Proton

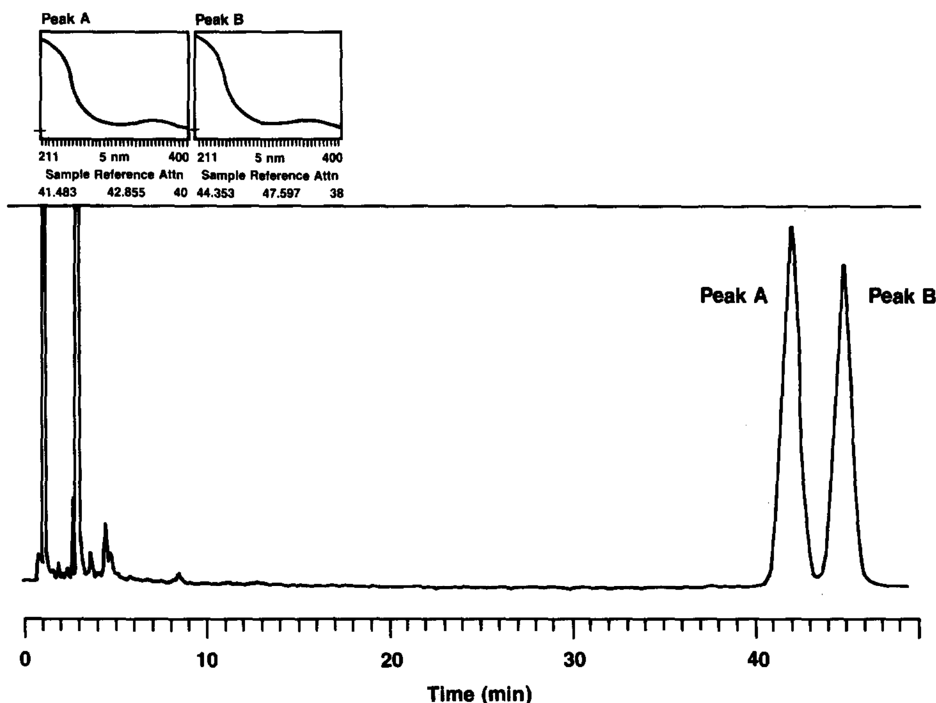


Fig. 1. Chromatographic resolution of the diastereomers of the bis-3,5-DNB derivative (7) on a 250 × 4.6 mm I.D. Spherisorb C_8, 5 μm column. Mobile phase: methanol–water (63:37, v/v). Flow-rate: 2.0 ml/min. UV spectra of peak A at 41 min and peak B at 44 min were obtained with an HP 1040A photodiode array detector at 254 nm.

TABLE I

RELATIVE RETENTION TIMES OF THE BIS-3,5-DNB DERIVATIVES OF THE OPTICAL ISOMERS OF RS-93522-004

Compounds	*RRT**	
	Peak A	Peak B
RS-93522-004	0.39	0.37
(*R*,*R*) isomer	0.40	
(*S*,*S*) isomer	0.40	
(*R*,*S*) isomer		0.38
(*S*,*R*) isomer		0.38

$$* \text{ Relative retention time} = \frac{\text{Retention time of compound 6}}{\text{Retention time of peak A or B}}$$

NMR and UV spectra (Fig. 1) confirmed the isolated compounds to be the diastereomers of the bis-3,5-DNB derivative of RS-93522-004. Negative-ion chemical ionization mass spectrometry using methane as the reagent gas gave a quasi-molecular ion $[M-2H]^-$ at m/z 912. The above spectral analyses of the two resolved peaks confirmed that the two are of the same chemical structure, suggesting the two peaks to be stereoisomers, namely diastereomers.

The elution order of the diastereomers of the bis-3,5-DNB derivative (7) was established by comparing the retention times with those of the bis-3,5-DNB derivatives of the individual optically active stereoisomers. Each individual isomer was derivatized and chromatographed using the described method. Since the mono-3,5-DNB derivative (6) was usually present in the derivatization mixture, it could be used as an internal reference peak for retention time comparison of the diastereomeric bis-3,5-DNB derivatives from different sample preparations. The relative retention time obtained for the bis-3,5-DNB derivatives of each optical isomer are in good agreement with the values obtained for RS-93522-004 (Table I). These data indicate that the order of elution is first the (*R*,*R*) (*S*,*S*) enantiomer pair and secondly the (*R*,*S*) (*S*,*R*) enantiomer pair.

Effect of yield on diastereomer ratio determination

Throughout the course of developing optimal reaction conditions, variable yields of the bis-3,5-DNB derivative were obtained. These yields ranged from approximately 28% to essentially quantitative. However, the diastereomer ratio of each RS-93522-004 sample determined by this method remained constant irrespective of reaction yield (Table II). These data indicate that the individual isomers react at the same rate during derivatization and that the diastereomer ratio determination is not affected by the reaction yield of the bis-3,5-DNB derivative and does not require quantitative derivatization.

Precision and accuracy

The precision of the chromatographic method was determined by performing six replicate injections of a reaction product from the 3,5-dinitrobenzoyl chloride

TABLE II

EFFECT OF YIELD ON DIASTEREOMER RATIO DETERMINATION

Yield (%)	*Peak A* (%)	*Peak B* (%)
28	52.82	47.18
40	52.92	47.08
54	52.45	47.55
100	53.25	46.75
100	53.06	46.94
100	52.38	47.62
Mean ± S.D.	52.81 ± 0.34	47.29 ± 0.34

derivatization of RS-93522-004. The ratios of the areas of the two diastereomer peaks (A:B) from the six injections gave a relative standard deviation of 0.06%, demonstrating the chromatographic method to be precise and repeatable.

The diastereomer ratio determination involves several steps, including derivatization and chromatography. In order to properly evaluate the accuracy of the method the products of six separate derivatization reactions of a single sample of RS-93522-004 were analyzed by HPLC. The diastereomer ratios from the six reactions resulted in a relative standard deviation of 0.68%, indicating that the analytical error associated with derivatization, reaction work-up, sample preparation and chromatography is not excessive.

Sensitivity

Under the given HPLC conditions, the diastereomers of the bis-3,5-DNB derivative separate with nearly baseline resolution, with a resolution factor ranging typically from 1.1 to 1.4. The varying degree of resolution reflects column to column variation. The sensitivity of the method was evaluated at a minimum resolution of 1.1 in two respects; first, the effect of injected sample amount on diastereomer ratio determination, and secondly, the detection limit of the (*R*,*R*) (*S*,*S*) diastereomer in the presence of the (*R*,*S*) (*S*,*R*) diastereomer and the converse. Solutions of the bis-3,5-DNB derivative, each containing 3.7, 4.4, 5.0 and 8.7 μg of 7 in a 25-μl injection volume were injected and gave the diastereomer ratios listed in Table III. The results indicate that within the concentration range examined the diastereomer ratio remains constant and is independent of sample amount injected.

TABLE III

EFFECT OF INJECTED SAMPLE AMOUNT ON DIASTEREOMER RATIO DETERMINATION

Sample amount (μg)	*Diastereomer ratio*
3.7	50.91 : 49.09
4.4	50.90 : 49.10
5.0	50.93 : 49.07
8.7	51.25 : 48.75

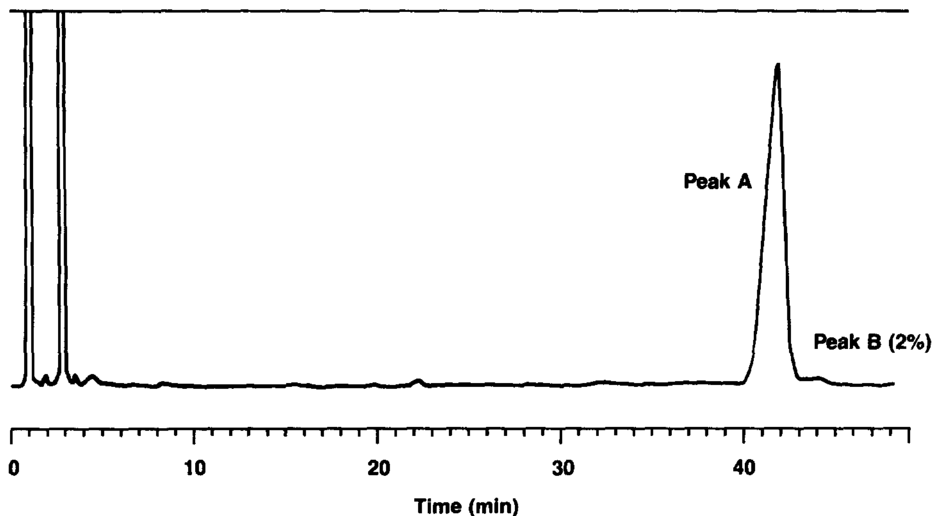


Fig. 2. Quantitation and chromatographic resolution of 2.0% (*R*,*S*) isomer in (*R*,*R*) isomer. Chromatographic conditions are the same as for Fig. 1.

The limit of detection was established for each diastereomer in a diastereomer mixture. Individual isomers, each containing a known small amount of the corresponding diastereomer, were derivatized and the detection limit for the minor diastereomer in the mixture was found to be about 0.3% for a typical 4-μg injection. Fig. 2 illustrates the quantitation of 2.0% of the (*R*,*S*) isomer in a sample of the (*R*,*R*) isomer. The method offers good sensitivity for the diastereomeric purity determination of RS-93522-004 and its stereoisomers.

CONCLUSION

We have described a simple procedure for the diastereomer ratio determination of a dihydropyridine-type calcium entry blocker, RS-93522-004, which contains a diol moiety. The procedure involves derivatization with 3,5-dinitrobenzoyl chloride followed by HPLC analysis of the resulting bis-3,5-dinitrobenzoate ester. The derivatization may be conveniently performed at a small (20 μmol) scale in a test tube with minimal sample work-up. The chromatography employs conventional reversed-phase HPLC on a C_8 column. The described procedure does not require quantitative derivatization. The reaction is very fast, being completed in 15 min and the analysis has been shown to be precise, accuate and sensitive.

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