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Separation of a New Antidiabetic Agent, N-(Trans-4-Isopropylcyclohexylcarbonyl)-D-Phenylalanine, and its Isomers by Chiral High-Performance Liquid Chromatography

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SEPARATION OF A NEW
ANTIDIABETIC AGENT,
N-(TRANS-4-ISOPROPYLCYCLO-
HEXYLCARBONYL)-D-PHENYLALANINE,
AND ITS ISOMERS BY CHIRAL
HIGH-PERFORMANCE LIQUID
CHROMATOGRAPHY

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ABSTRACT

N-(*trans*-4-Isopropylcyclohexylcarbonyl)-D-phenylalanine (A4166) is a new oral antidiabetic agent. In order to determine the purity of chemical samples of A4166, a high-performance liquid chromatographic method for the separation of A4166 and synthetic by-products (an L-enantiomer and a *cis*-isomer of A4166) has been developed. A chiral stationary phase column packed with 5 μ m N-(*tert*-butylaminocarbonyl)-L-valylaminopropyl silica gel was used for the direct separation of A4166 and its isomers after derivatization with a non-chiral reagent.

INTRODUCTION

N-(*trans*-4-Isopropylcyclohexylcarbonyl)-D-phenylalanine (A4166) is a new oral antidiabetic agent which exhibits a significant blood glucose lowering activity (1, 2, 3). The structure is shown in Figure 1. In the preparation of A4166, N-(*cis*-4-isopropylcyclohexylcarbonyl)-D-

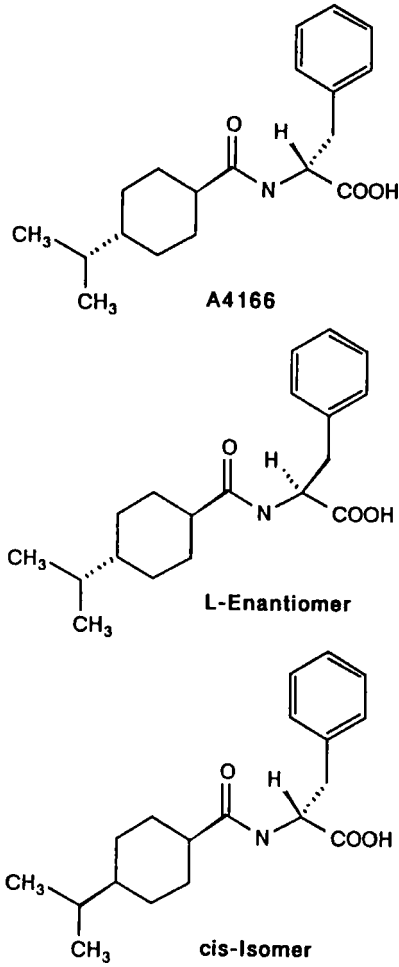


Figure 1. Chemical structures of A4166, the L-enantiomer and the cis-isomer.

phenylalanine (*cis*-isomer) and N-(*trans*-4-isopropylcyclohexylcarbonyl)-L-phenylalanine (L-enantiomer) are produced as by-products. A4166 can be purified by recrystallization. It would be advantageous to be able to monitor the purity of recrystallized A4166. Also, A4166 is a much more potent hypoglycemic agent than its isomers and it is necessary to avoid contamination with the isomers. A4166 exhibits a 20 % blood glucose decrease at an oral dose of 1.6 mg / kg; however, its isomers require a dose of about 100 mg / kg to show equal potency. This report details how the separation of the three closely related isomers (A4166, *cis*-isomer and L-enantiomer) as *tert*-butyl esters has been achieved by HPLC using a chiral stationary phase column packed with 5 μ m N-(*tert*-butylaminocarbonyl)-L-valylaminopropyl silica gel and a ternary solvent mixture of *n*-hexane / 1,2-dichloroethane / ethanol (100 / 20 / 0.05). The method gave good separation and reproducibility of the analysis of the mixture of A4166, *cis*-isomer and L-enantiomer.

MATERIALS AND METHOD

Chromatographic Conditions

The chromatography was performed with a Model 655A-12 liquid chromatograph (Hitachi, Tokyo, Japan) equipped with a Model 7125 syringe-loading injector (Rheodyne, California, USA), a Model 655A-21 variable wavelength UV monitor (Hitachi, Tokyo, Japan) set at 220 nm and a Model 655-71 data processor (Hitachi, Tokyo, Japan). The column was a SUMIPAX OA-3000 (25 cm x 4 mm I.D.) packed with 5 μ m N-(*tert*-butylaminocarbonyl)-L-valylaminopropyl silica gel. *n*-Hexane / 1,2-dichloroethane / ethanol mixture was used as the mobile phase. A flow-rate of 1.0 mL / min was typically used at room temperature.

Chemicals and Reagents

All solvents, *n*-hexane, 1,2-dichloroethane and ethanol were of guaranteed grade and purchased from Wako Chemicals (Osaka, Japan). Individual samples of A4166, *cis*-isomer, L-enantiomer, and their esters were synthesized in our laboratory. The esterification reagent, N,N-dimethylformamide di-*tert*-butyl acetal was purchased from Wako Chemicals (Osaka, Japan).

Sample Preparation

Standard solution for calibration and quantitation were prepared by dilution of 1.0 mg / mL stock solutions of A4166, the *cis*-isomer and the L-enantiomer. All solutions were prepared in 1,2-dichloroethane. 1 mL of the above solution was concentrated. 100 μ l of N,N-dimethylformamide di-*tert*-butyl acetal (4, 5) was added to the residue. The mixture was heated at 60 °C for 20 min, cooled and diluted to 1 mL with 1,2-dichloroethane. A 20 μ l sample solution was injected into the chromatograph.

RESULTS AND DISCUSSION

Methyl, *tert*-butyl and benzyl esters of A4166 and its isomers were synthesized and their separation attempted. The enantiomers of the methyl ester and benzyl ester gave poor peak separation. A measurable separation was achieved for the *tert*-butyl esters of A4166 and its isomers.

The reactivities and the time courses of the derivatization with N,N-dimethylformamide di-*tert*-butyl acetal were investigated for A4166, the L-enantiomer and the *cis*-isomer. Solutions of the synthetic esters were

used as references for the quantitation of the reaction products (e.g. 100 μg A4166 = 118 μg of the ester). It was found that the reaction, assessed by comparison of peak heights, was complete almost quantitatively within 20 min. The three isomers were allowed to react separately, each compound produced a single chromatographic peak, demonstrating the reactions proceed without racemization.

It was found that the A4166 and its isomers peaks showed considerable tailing when an *n*-hexane / 1,2-dichloroethane mobile phase was employed. The tailing caused the unsatisfactory separation of peaks. To alleviate the tailing problem, ethanol was added to the mobile phase. The use of ethanol in the mobile phase improved peak tailing and resolution. Various combinations of a ternary solvent system, *n*-hexane / 1,2-dichloroethane / ethanol, were studied. Optimal separation of these three isomers was achieved using a 100 / 20 / 0.05 volume ratio of *n*-hexane / 1,2-dichloroethane / ethanol at a flow rate of 1 mL / min. A base line separation was achieved for these compounds. The capacity factors (k') for the *cis*-isomer, A4166, and the L-enantiomer were determined as 1.03, 1.30 and 1.65, respectively. The separation factors were 1.26 (α_{12}) and 1.27 (α_{23}) (Figure 2).

Calibration curves of A4166, *cis*-isomer and L-enantiomer after derivatization with N,N-dimethylformamide di-*tert*-butyl acetal were constructed over the range 1.0 - 1000.0 μg / mL for each isomers. Regression data for peak area and peak height responses are shown in Table 1.

Three separate stock solutions of A4166, the L-enantiomer and the *cis*-isomer were used to prepare 90-5-5, 96-2-2 and 98-1-1 percent mixtures,

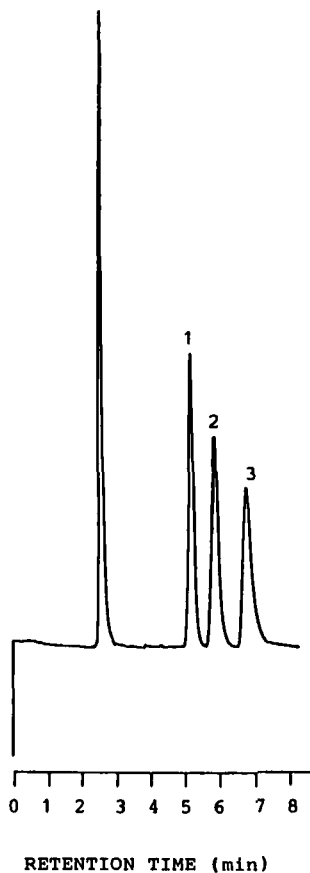


Figure 2. Chromatogram of A4166 and its isomers.

1 = *cis*-isomer; 2 = A4166; 3 = L-enantiomer.

respectively. These samples were derivatized by the previous procedure. Values for precision and accuracy were estimated with the three samples (Table 2). The coefficient of variation ranged from 1.1 to 7.1 % with an average of 4.1 %. The precision analysis indicated that good reproducibility is possible with reasonable precision and acceptable accuracy.

TABLE 1.

Regression Data for A4166, L-Enantiomer and cis-Isomer

	Range ($\mu\text{g/mL}$)	Intercept	Slope	Correlation coefficient
A4166				
peak height	1.0 - 1000.0	0.00	0.45	0.999
peak area	1.0 - 1000.0	0.00	0.59	0.999
L-Enantiomer				
peak height	1.0 - 1000.0	0.00	0.33	0.999
peak area	1.0 - 1000.0	0.00	0.61	0.999
cis-isomer				
peak height	1.0 - 1000.0	0.00	0.57	0.999
peak area	1.0 - 1000.0	0.00	0.61	0.999

TABLE 2.

The Precision and Accuracy of the HPLC method

% A4166	% L-enantiomer	% cis-isomer	Mean \pm SD*		
			A4166	L-enantiomer	cis-isomer
90	5	5	88.7 \pm 1.24	4.93 \pm 0.24	5.11 \pm 0.27
96	2	2	95.2 \pm 1.33	1.91 \pm 0.09	2.06 \pm 1.03
98	1	1	97.0 \pm 1.10	0.98 \pm 0.07	1.20 \pm 0.07

*n = 5

The evaluated chromatographic conditions were used to generate percent purity values for the three A4166 lots. These samples were derivatized by the previous procedure. In these samples, impurities were not detected. The mean percent purity values for the three lots were 99.5 %, 101.2 % and 101.0 %. The mean values are the average for five determinations. The experimental values are based on the response factors of the A4166, the *cis*-isomer and the L-enantiomer standards. Relative standard deviation values for the percent purity determinations ranged from 0.96 to 1.27.

CONCLUSIONS

The HPLC method for separation of the three closely related isomers as a *tert*-butyl ester was developed. The developed method gives quantitative measurement of each isomer, and has been validated for generation of quantitative data for A4166 lots with external standards.

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