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ANALYSIS OF ENANTIOMERS OF A NEW ANTIDIABETIC AGENT IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A new antidiabetic agent, N-(*trans*-4-isopropylcyclohexylcarbonyl)-D-phenylalanine (A4166), and its L-enantiomer were successfully separated and quantified by high-performance liquid chromatograph. This direct resolution was accomplished using a chiral stationary phase column packed with 5 μm N-(*tert*-butylaminocarbonyl)-L-valylaminopropyl silica gel and a mobile phase consisting of *n*-hexane / *n*-propanol / trifluoroacetic acid. The method has been used for the analysis of plasma samples from beagle dogs.

INTRODUCTION

A4166, N-(*trans*-4-isopropylcyclohexylcarbonyl)-D-phenylalanine, is a new orally antidiabetic agent with a chiral center in the α -position to the

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carboxyl group (Figure 1). A4166 and its L-enantiomer, N-(*trans*-4-isopropylcyclohexylcarbonyl)-L-phenylalanine, are stereospecific in action (1, 2, 3). A4166 is a much more potent hypoglycemic agent than its L-enantiomer. A4166 exhibits a 20% blood glucose decrease at an oral dose of 1.6 mg / kg; however, its L-enantiomer requires a dose of more than 100 mg / kg to show equal potency. Stereoselective action of enantiomers results in the difference of not only intrinsic activity and receptor-binding properties but also pharmacokinetics. Enantiomers often show different pharmacokinetics behavior (4), so it is necessary to determine the pharmacokinetics of individual enantiomers. However, there is no information on the pharmacokinetics of individual enantiomers in man or in animals after administration. Drugs with a chiral center in the α -position to the carboxyl group were found to be converted in vivo into the corresponding enantiomers (5,6,7). Therefore, a method that can discriminate between enantiomers is needed in order to obtain information on the pharmacokinetics of individual enantiomers and the racemization of A4166.

The direct stereoisomeric separation and quantitation of A4166 and its L-enantiomer was accomplished in this study using a chiral stationary phase column packed with 5 μ m N-(*tert*-butylaminocarbonyl)-L-valylaminopropyl silica gel. Sample preparation involves the use of solid-phase extraction on a Sep-pak C₁₈ cartridge column with N-(*trans*-4-methylcyclohexylcarbonyl)-D-phenylalanine (Figure 1) added as the internal standard. A simple and specific plasma analysis for the quantitative measurement of the enantiomers was developed based on the established separation system.

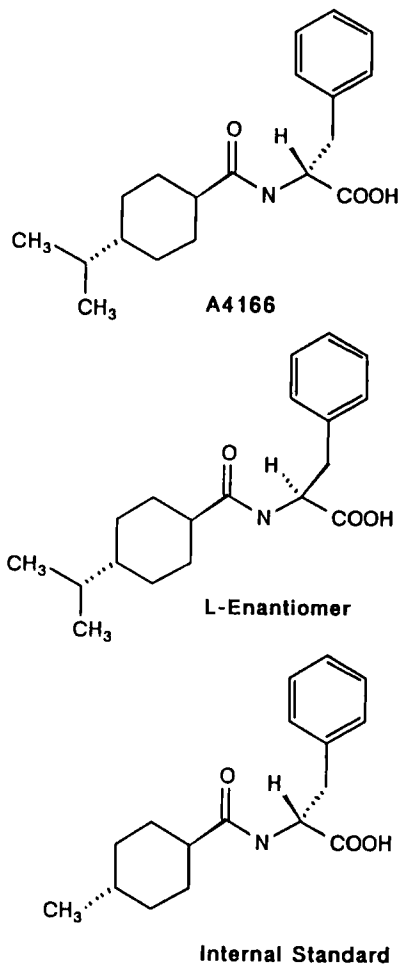


Figure 1. Chemical structures of A4166, the L-enantiomer and the internal standard.

EXPERIMENTAL

Apparatus

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 254 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 μm N-(*tert*-butylaminocarbonyl)-L-valylaminopropyl silica gel. An *n*-Hexane / *n*-propanol / trifluoroacetic acid mixture was used as the mobile phase. A flow-rate of 0.5 mL / min was typically used at room temperature.

Chemicals and Reagents

A4166, the L-enantiomer and the internal standard were synthesized in our laboratory. All solvents, *n*-hexane, *n*-propanol and trifluoroacetic acid were of HPLC grade and purchased from Wako Chemicals (Osaka, Japan).

Sample Preparation

The stock solution of A4166 (1 mg / mL), the L-enantiomer (1 mg / mL) and the internal standard (1 mg / mL) were prepared by dissolving accurately weighed samples in *n*-propanol. Working solution of appropriate concentrations were made every week by dilution of the stock solutions with *n*-propanol. The calibration curve standards were prepared by adding known amounts of A4166, the L-enantiomer and the internal standard to blank plasma, and contained 2 - 30 μg / mL of A4166, 2 - 30 μg / mL of the L-enantiomer and 10 μg / mL of the internal standard.

Sample Work-up

A 200 μl volume of the internal standard (25 μg / mL) and 2 mL of 0.1 M phosphate buffer (pH 6.6) were added to 0.5 mL of plasma. Plasma

samples were adsorbed on a disposable reversed phase extraction column, Sep-pak C₁₈ (Waters). The column was conditioned twice with 3 mL of methanol, twice with 3 mL of water and once with 2 mL of 0.1 M phosphate buffer (pH 6.6). The sample solution was forced by a syringe through the column, followed by 15 mL volumes of water. The sample-containing fraction was eluted with 2 mL of methanol. This solution was evaporated under vacuum. The residue was dissolved in 0.5 mL of the mobile phase and 20 μ l was injected onto the column.

RESULTS AND DISCUSSION

The high-performance liquid chromatographic chiral stationary phases have been successful in the direct enantiomeric resolution of a variety of chiral molecules. However, carboxylic acids are typically difficult to resolve. Enantiomers of carboxylic acids are generally separated by the chiral stationary phase column after derivatization to their corresponding amides and esters. It was found that carboxylic acid enantiomers, A4166 and its L-enantiomer, were directly separated by the use of trifluoroacetic acid in the mobile phase. The optimal separation of A4166 and its enantiomer was performed on the chiral stationary phase column with a ternary solvent system, *n*-hexane / *n*-propanol / trifluoroacetic acid (97.5 / 2.5 / 0.1) at a flow rate of 0.5 mL / min. The detection wavelength was set to 254 nm. Under these conditions, the capacity factors (k') for A4166 and its L-enantiomer were determined as 3.54 and 3.90, respectively. The separation factor (α) was 1.10 (Figure 2).

Values for precision and accuracy were estimated at 2, 4, 10, 20 and 30 μ g / mL A4166 and its L-enantiomer in plasma are presented in Table

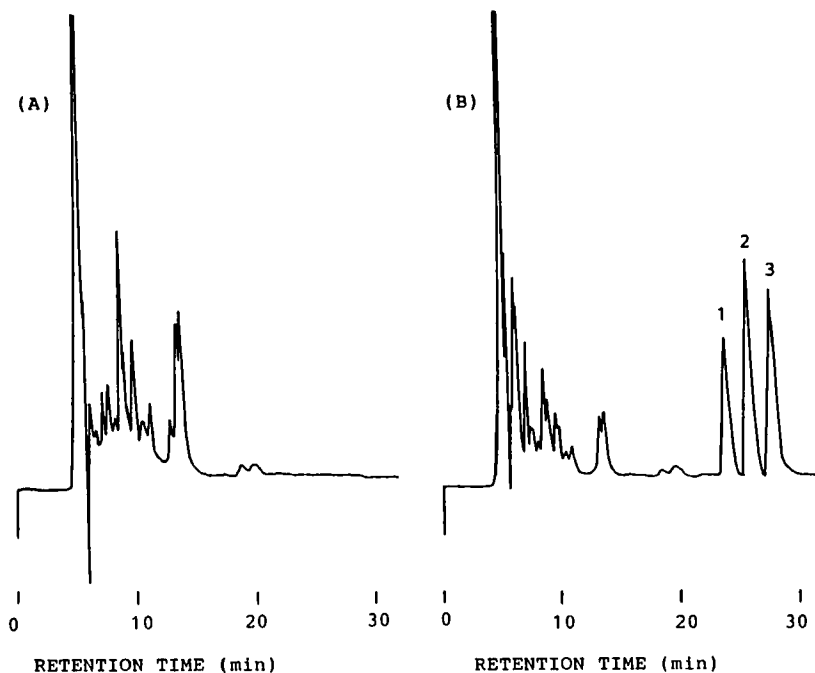


Figure 2. Typical chromatograms of (A) extract of a blank dog plasma sample and (B) extract of a dog plasma sample obtained after oral administration of the mixture of A4166 (10 mg / kg) and its L-enantiomer (10 mg / kg).

1 = L-Enantiomer; 2 = A4166; 3 = Internal standard.

1. The coefficient of variation ranged from 0.8 to 3.1 % with an average of 1.7 %.

Calibration curves of A4166 and its enantiomer were constructed over the range 2 - 30 $\mu\text{g} / \text{mL}$ for each isomer by comparison of the peak height of each enantiomer with those of the internal standard.

Regression data for peak height responses are shown in Table 2.

To determine recovery, the peak height of the extraction of plasma samples spiked with A4166 or its L-enantiomer were compared with the

TABLE 1
The Precision and Accuracy of the HPLC Method

Concentration ($\mu\text{g} / \text{mL}$)	Enantiomer	Detected concentration ¹	Coefficient of variation (%)
2	D	1.90 ± 0.05	2.6
	L	1.92 ± 0.06	3.1
4	D	4.04 ± 0.10	2.5
	L	4.10 ± 0.08	2.0
10	D	9.89 ± 0.11	1.1
	L	10.13 ± 0.08	0.8
20	D	19.97 ± 0.15	0.8
	L	20.33 ± 0.24	1.2
30	D	30.05 ± 0.47	1.6
	L	29.96 ± 0.33	1.1

¹Mean \pm SD, n = 5

TABLE 2.
Regression Data for A4166 and the L-Enantiomer

	Range ($\mu\text{g} / \text{mL}$)	Intercept	Slope	Correlation coefficient
A4166	2 - 30	0.000	1.03	0.999
L-Enantiomer	2 - 30	0.000	0.98	0.999

same amount of drugs as standard solution injected directly onto the column without extraction. The percent recoveries of A4166 and its L-enantiomer from the C₁₈ extraction column are shown in Table 3.

The utility of the assay was demonstrated by analyzing A4166 and the L-enantiomer in plasma samples from beagle dogs. A mixture of A4166 (10 mg / kg) and the L-enantiomer (10 mg / kg) was given to the dogs. Typical chromatograms are shown in Figure 2. Figure 3 shows plasma concentration-time profiles. The mean concentrations of the A4166 (D-enantiomer) were higher than those of the L-enantiomer. However, this difference of absorption is not enough to explain the fact that the D-enantiomer (A4166) showed a 100-fold higher activity than the L-enantiomer. To determine whether racemization occurs in vivo, a plasma sample obtained after administration of a single oral dose of 10 mg / kg of A4166 was analyzed (Figure 4). The L-enantiomer was not detected. In this assay, the racemization of A4166 in vivo was not observed.

TABLE 3.
Recovery from Solid Phase Extraction (Sep-pak C₁₈)

Sample		% Recovery ¹
A4166	4 µg / mL	95.7 ± 2.6
	20 µg / mL	95.4 ± 1.2
L-Enantiomer	4 µg / mL	96.5 ± 2.9
	20 µg / ml	96.2 ± 0.8

¹Mean ± SD, n = 3

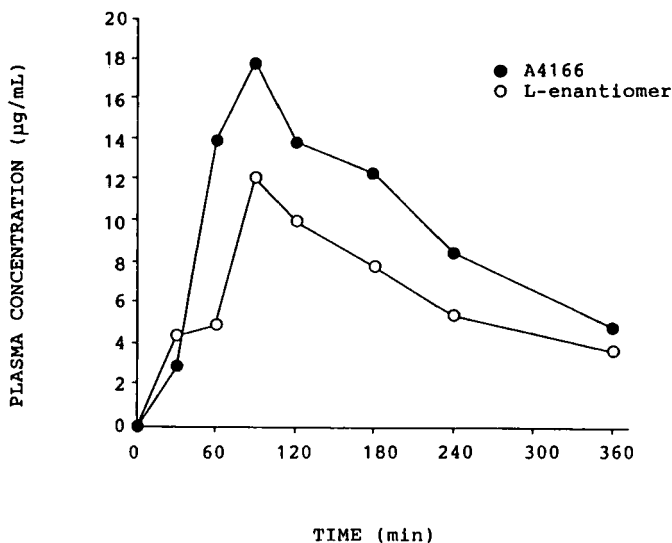


Figure 3. Mean curve of the concentrations of enantiomers obtained after oral administration of the mixture of A4166 (10 mg / kg) and its L-enantiomer (10 mg / kg).

CONCLUSIONS

A liquid chromatographic procedure has been described for the separation and quantitation of A4166 and its L-enantiomer. These compounds can be isolated from plasma in high yield using a reversed phase (C_{18}) solid extraction procedure. The assay is sufficiently rapid and simple to allow accurate and precise measurements of plasma levels of A4166 and its L-enantiomer during pharmacokinetic studies in dogs.

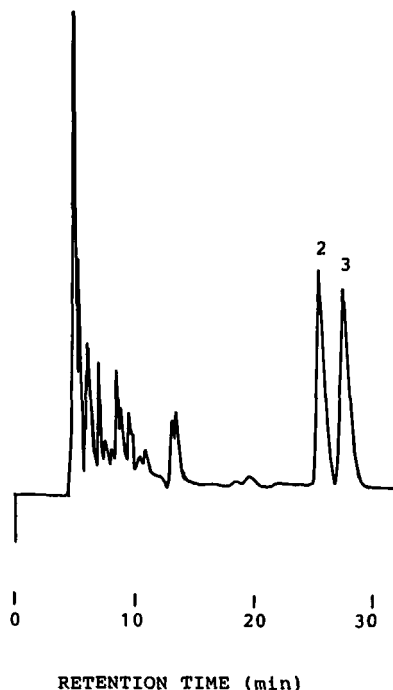


Figure 4. Chromatogram of plasma samples obtained 180 min after administration of a single oral dose of 10 mg / kg of A4166. 2 = A4166; 3 = Internal standard.

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