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Short communication

Determination of N-(*trans*-4-isopropylcyclohexylcarbonyl)-D-phenylalanine in human plasma by solid-phase extraction and column-switching high-performance liquid chromatography with ultraviolet detection

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Abstract

A column-switching high-performance liquid chromatography method with ultraviolet detection at 210 nm has been developed for the determination of N-(*trans*-4-isopropylcyclohexylcarbonyl)-D-phenylalanine (AY4166, I) in human plasma. Plasma samples were prepared by solid-phase extraction with Sep-Pak Light tC_{18}, followed by HPLC. The calibration graph for I was linear in the range 0.1–20 μg/ml. The limit of quantitation of I, in plasma, was 0.05 μg/ml. The recovery of spiked I (0.5 μg/ml) to drug-free plasma was over 92% and the relative standard deviation of spiked I (0.5 μg/ml) compared to drug-free plasma was 4.3% (n = 8).

Keywords: N-(*trans*-4-Isopropylcyclohexylcarbonyl)-D-phenylalanine

1. Introduction

Numerous techniques, including pre- and post-column labelling and column-switching, have been developed in order to extend the application of high-performance liquid chromatography (HPLC). Column-switching is a simple, rapid technique that has been applied to complex sample matrices, such as the analysis of drugs as well as their metabolites in biological fluids [1–5].

N-(*trans*-4-Isopropylcyclohexylcarbonyl)-D-phenylalanine (AY4166, I) (Ajinomoto, Tokyo and Yamanouchi, Tokyo) is a new amino acid derivative with hypoglycaemic activity and clinical evaluation of the drug for its hypoglycaemic effect has been carried out.

In previous papers [5–8], various assay methods were developed for the determination of I and its related compounds, in animal plasma and urine. However, when we applied these methods to human plasma samples during clinical studies, these methods showed poor separation and low sensitivity because of irregularities of the baselines and tailing of the peaks on the chromatogram for each compound. Pharmacokinetic and toxicokinetic studies on the administration of I required a sensitive assay

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method, capable of quantifying concentrations of 0.1 μg/ml or less, for I in physiological fluid.

In this paper, we report a simple, rapid and highly sensitive method for the determination of I in human plasma by column-switching HPLC with ultraviolet detection at 210 nm, after solid-phase extraction.

2. Experimental

2.1. Reagents and materials

Compound I and N-(*trans*-4-*tert*.-butylcyclohexylcarbonyl)-D-phenylalanine (A-4263, internal standard) were synthesized and supplied by our laboratories. Acetonitrile and methanol were both of HPLC grade and obtained from Wako (Osaka, Japan) and Junsei (Tokyo, Japan), respectively, and other reagents were all of analytical reagent grade. Sep-Pak Light tC_{18} cartridges (130 mg/3 ml) were purchased from Waters (Milford, MA, USA). The cartridges were used after successive washing with ethanol (5 ml), purified water (5 ml) and 0.05 *M* sodium phosphate buffer (pH 6.6) (5 ml).

2.2. Internal standard preparation

The internal standard was dissolved in methanol and diluted to 80 μg/ml with acetonitrile–0.05 *M* sodium phosphate buffer, pH 6.6 (36:64, v/v), in a volumetric flask. The internal standard solution was stable in methanol for more than one month at 4°C.

2.3. Apparatus and conditions

An LC-10A system liquid chromatograph equipped with a FCU-12AH column-switching device (Shimadzu, Kyoto, Japan) was used. The detector wavelength was set at 210 nm. The sample was applied using a Rheodyne Model 7125 sample loop injector with an effective volume of 20 μl. HPLC was carried out on an L-Column ODS (5 μm) analytical column (250 × 4.6 mm, I.D.) (Chemicals Inspection and Testing Institute, Tokyo, Japan) and an Inertsil ODS-2 (5 μm) precolumn (10 × 4 mm, I.D.) (GL Science, Tokyo, Japan) using acetonitrile–0.05 *M* phosphate buffer, pH 6.6 (36:64, v/v) as the solvent system for assaying (solution A) and acetonitrile–0.05 *M* sodium phosphate buffer, pH 6.6 (60;40, v/v) as the solvent system for cleaning the precolumn (solution B). The flow-rate was 1.0 ml/min at 50°C.

The HPLC system had been equilibrated previously with solution A and a 20-μl sample solution was injected into the precolumn. After 0.8 min, the substances were introduced into the analytical column and the precolumn was washed with solution B, by switching the six-port valve. After 20 min, the precolumn was equilibrated with solution A for 10 min by returning the six-port valve to its original position.

2.4. Plasma collection

Blood was collected freshly in a heparinized vacutainer tube from healthy individuals and drug-free plasma was obtained by centrifugation at 1700 *g* for 15 min at 5°C. The obtained plasma was then used immediately for the determination of I.

2.5. Sample preparation

To 0.5 ml of plasma in an Eppendorf tube, 0.05 ml of internal standard and then 0.5 ml of 0.05 *M* sodium phosphate buffer (pH 6.0) were added. The mixture was vortex-mixed for 10 s and applied to a Sep-Pak Light tC_{18} that had been pretreated with 5 ml of ethanol, 5 ml of water and 5 ml of 0.05 *M* sodium phosphate buffer (pH 6.0). The cartridge was washed with 2 ml of 0.05 *M* sodium phosphate buffer (pH 6.0) and 1 ml of water and finally was eluted with 2 ml of ethanol. The eluate was evaporated to dryness in vacuo at 30°C. The residue was dissolved in 0.2 ml of solution B and an aliquot containing 20 μl of solution was injected into the HPLC system.

3. Results and discussion

3.1. Chromatography

Our first effort focused on the analysis of I in human plasma using column-switching HPLC with UV detection at 210 nm, following solid-phase extraction. I in plasma was determined by HPLC

with solid-phase extraction using Sep-Pak C_{18} [6], but this method did not give a clear separation of the I peak from trace amounts of interference peaks in pretreated plasma and did not have the sensitivity required to determine the concentration of I in plasma at levels of 0.1 μg/ml or less. In order to develop a new, sensitive assay method, the separation of I from interfering substances in pretreated samples and the removal of the late-eluted materials from the column were examined.

At the beginning of the work, column-switching times from the pre-column to the analytical column were examined, to determine their effect on the analysis of I and the elimination of interferences caused by the complex sample matrices. The column-switching HPLC was equipped with an L-Column ODS column as an analytical column and with an Inertsil ODS-II column as the precolumn.

Fig. 1 shows the relationship between detector responses and column-switching times. The detector responses of I and internal standard reached constant levels at 0.5 and 0.6 min, respectively. When the column-switching time was made longer than 0.8 min, many unknown peaks were observed and I could not be identified. Thus, the column-switching time was set at 0.8 min. Chromatograms of I in human plasma, obtained by column-switching HPLC with detection at 210 nm after solid-phase extraction, are shown in Fig. 2. The peak of I was separated completely from other peaks. The peaks of I and the internal standard were observed at the retention times of ca. 11.7 and 16.2 min, respectively.

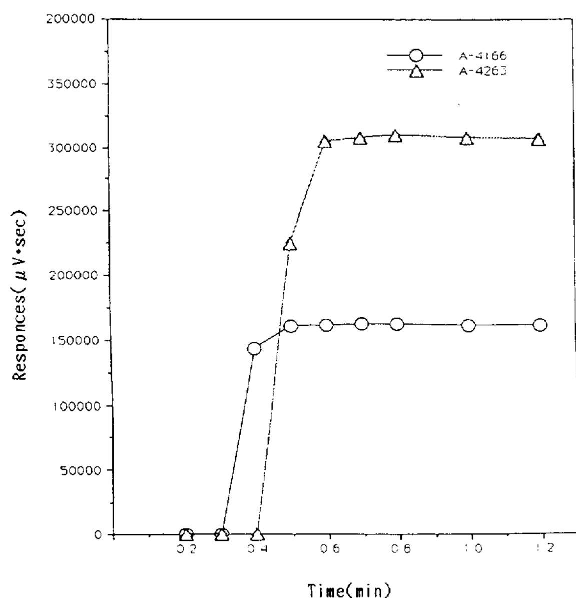

Fig. 1. Relationship between detector responses and column-switching time.

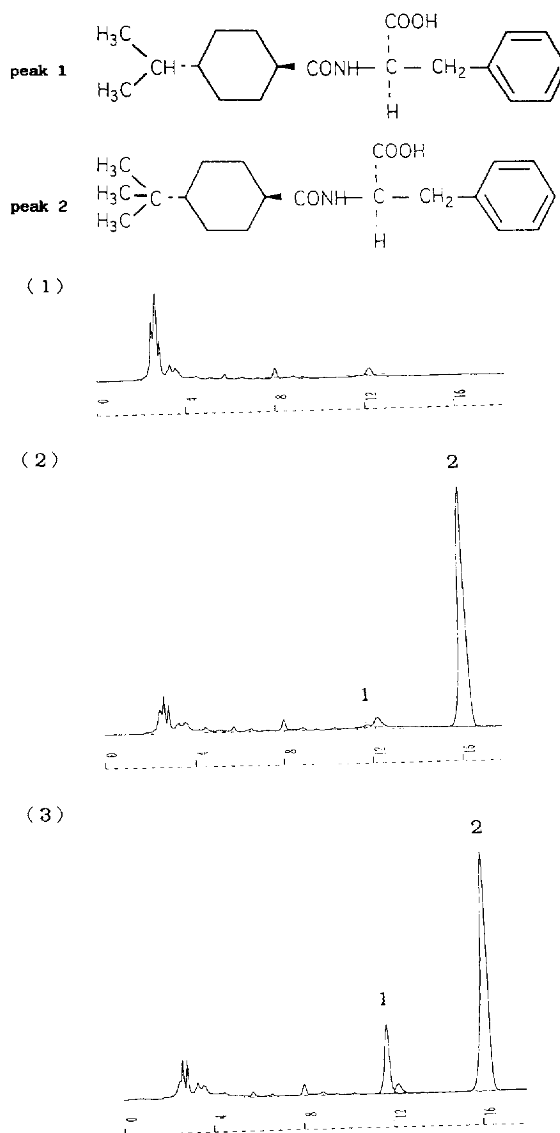

Fig. 2. Chromatograms of I in human plasma by column-switching HPLC. 1 = Blank human plasma; 2 = human plasma spiked with I (0.05 μg/ml); 3 = human plasma spiked with I (2.0 μg/ml).

3.2. Sample preparation

Our second effort focused on the examination of the cartridges for solid-phase extraction.

When the previous preparation method for dog plasma using Sep-Pak C_{18} as the solid-phase extraction cartridge [6] was used to determine trace amounts of I in human plasma, the quantitation of I was found to be impossible, because of the interferences and the insoluble materials from the cartridge. Many kinds of disposable cartridges were examined. The unknown substances and interferences were removed well by the Sep-Pak Light tC_{18} cartridge compared to other cartridges. The Sep-Pak Light tC_{18} was consequently selected as the new solid-phase extraction cartridge. This cartridge was washed with 5 ml of ethanol and then with 5 ml of 0.05 *M* sodium phosphate buffer (pH 6.0) before use.

The sample preparations were carried out as follows: the sample solution (0.5 ml) was applied to the cartridge followed by washing with 2 ml of a mixture containing acetonitrile–0.5 *M* sodium phosphate buffer, pH 6.0 (20:80, v/v) and was eluted with 2 ml of methanol. The eluate was concentrated to dryness in vacuo at 30°C and the residue was dissolved in solution A.

Table 1
Recoveries and R.S.D. of I added to human plasma

I (μg/ml)		Recovery (%)	R.S.D. (%)
Added	Found		
0.50	0.46	92	4.3[a]
2.00	1.88	94	–
5.00	4.75	95	2.4[b]
20.0	19.2	96	0.6[b]

[a] $n = 8$.
[b] $n = 6$.

3.3. Determination of I

For the generation of calibration graphs, peak-area ratios of I to internal standard were plotted against concentration. Over the concentration range 0.05–20 μg/ml of human plasma, calibration graphs were found to be linear with correlation efficients greater than 0.999 ($y = 4.8081x - 0.052676$). The recovery of I was greater than 92% and the relative standard deviation (R.S.D.) was 4.3% at 0.5 μg/ml spiked in human plasma as shown in Table 1.

4. Conclusion

The proposed method using solid-phase extraction and column-switching HPLC is a rapid, simple, highly sensitive and reproducible method for the determination of I in human plasma. This method is applicable to lower levels of I in human plasma and the measurement of many samples for toxicokinetic and pharmacokinetic studies were carried out. The analysis time of I in human plasma was 30 min and the limit of quantitation in human plasma was 0.05 μg/ml.

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