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Determination of *N*-(*trans*-4-isopropylcyclohexanecarbonyl)-D-phenylalanine and its metabolites in human plasma and urine by column-switching high-performance liquid chromatography with ultraviolet detection

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Abstract

A simple, rapid and sensitive two column-switching high-performance liquid chromatographic (HPLC) method with ultraviolet detection at 210 nm has been developed for the determination of *N*-(*trans*-4-isopropylcyclohexanecarbonyl)-D-phenylalanine (AY4166, I) and its seven metabolites in human plasma and urine. Measurements of I and its metabolites were carried out by two column-switching HPLC, because metabolites were classified into two groups according to their retention times. After purification of plasma samples using solid-phase extraction and direct dilution of urinary samples, I and each metabolite were injected into HPLC. The calibration graphs for plasma and urinary samples were linear in the ranges 0.1 to 10 μg ml^{-1} and 0.5 to 50 μg ml^{-1}, respectively. Recoveries of I and its seven metabolites were over 88% by the standard addition method and the relative standard deviations of I and its metabolites were 1–6%.

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

1. Introduction

N - (*trans* - 4 - Isopropylcyclohexanecarbonyl) - D - phenylalanine (AY4166, I) is a new amino acid derivative with hypoglycaemic activity and clinical evaluation for hyperglycaemic effect has been carried out [1–5]. Ten metabolites (M1 to M10) of I have been isolated from animal urine and bile and characterized (Fig. 1) [6]. M1, M2 and M3 are hydroxy substituents of isopropyl group of I. M4, M5 and M6 are glucuronic acid conjugates of I. M7 is a derivative of I with the isopropyl group change to isopropenyl group. M8 is a D-tyrosine derivative instead of D-phenylalanine. M9 and M10 are carboxy substituents of isopropyl group of I. M1–M7 are detected as major metabolites in human plasma and urine. On the other hand, M8–M10 were rarely detected in human plasma and urine.

Pharmacokinetic and toxicokinetic studies on the administration of I required a simple, rapid and highly sensitive assay method capable of quantifying trace amounts of metabolites of I. Solid-phase extraction (SPE) and column-switching high-performance liquid chromatography (HPLC) were useful

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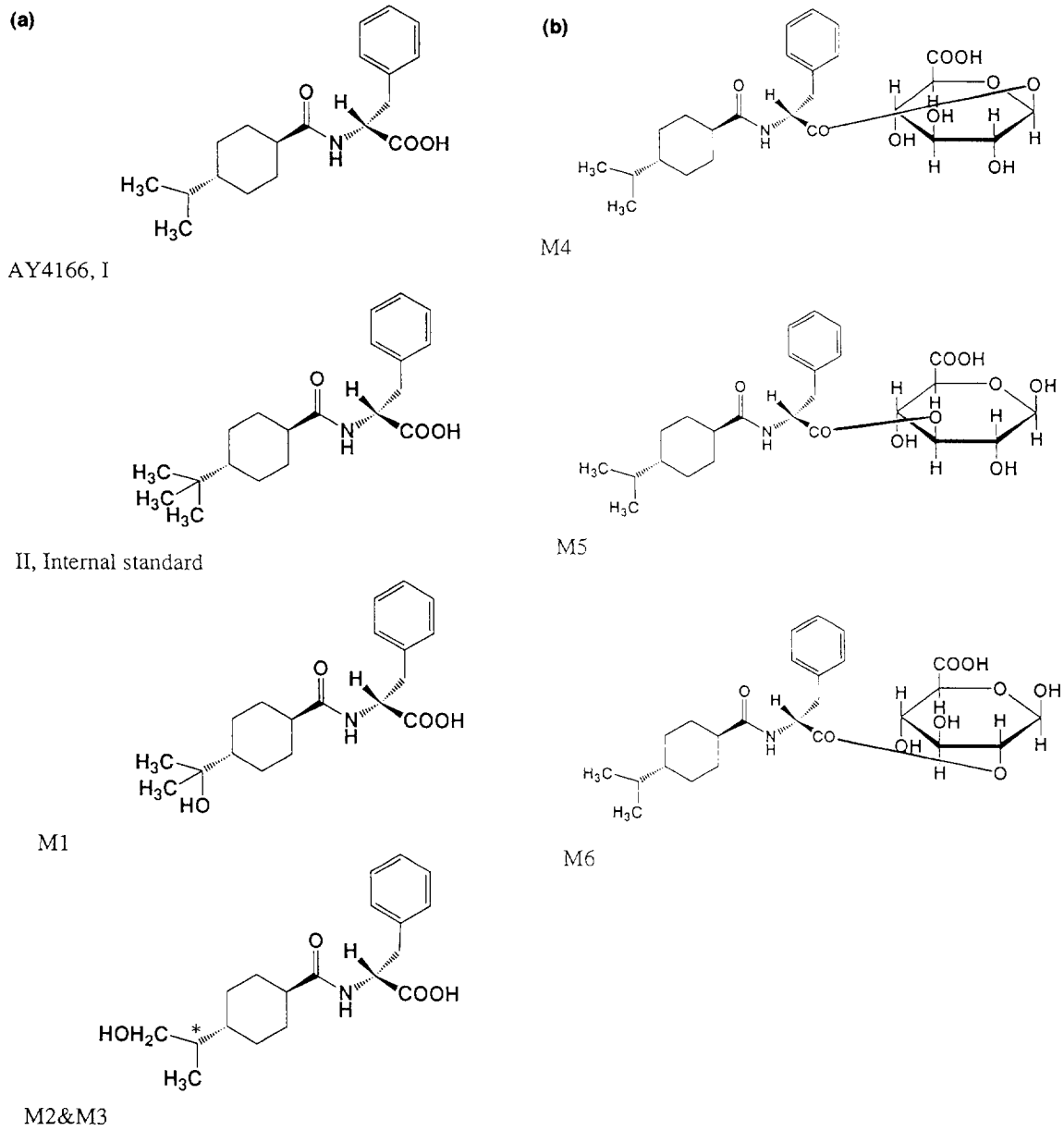

Fig. 1. Structures of I and its metabolites.

methods to detect trace amounts of components in biological samples [4,5,7–10]. A simultaneous determination of I and its metabolites in both plasma and urine was designed by column-switching HPLC using SPE as purification for plasma samples and direct dilution as pretreatment for urinary samples.

In the present paper, the SPE method was used for plasma samples and direct dilution method was used

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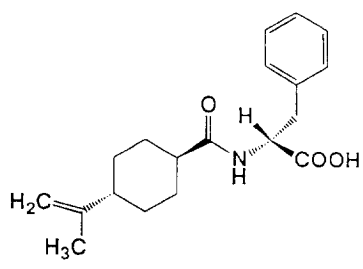
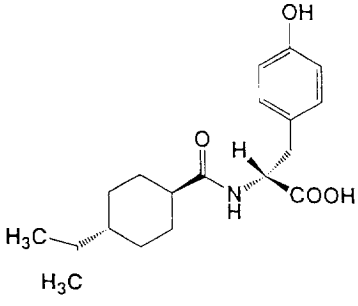
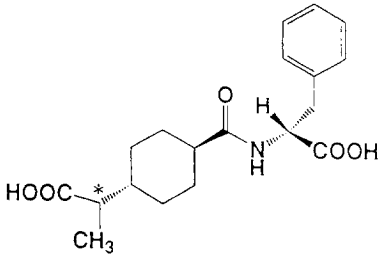


M7



M8



M9&M10

Fig. 1. (*continued*)

for urinary samples and the measurements of I and its metabolites were carried out by two column-switching HPLC using ultraviolet (UV) detection at 210 nm, because I and its metabolites were classified into two groups according to their retention times.

Furthermore, this paper also deals with the validity of the determination of I and its metabolites in both human plasma and urinary samples by the method proposed here and its applicability to simultaneous determination of lower levels of I and its metabolites in human plasma and urine.

2. Experimental

2.1. Reagents and materials

I, M1, M2, M3, M7 and *N*-(*trans*-4-*tert*.-butylcyclohexanecarbonyl)-D-phenylalanine (A-4263, II, internal standard) were synthesized and supplied by our laboratories. M4, M5 and M6 were fractionated from human urine after administration of I according to the literature [6]. Acetonitrile was of HPLC grade from Wako (Osaka, Japan) and other reagents were all of analytical reagent grade. Sep-Pak Vac tC_{18} cartridge (200 mg/3 ml) were purchased from Waters (Milford, MA, USA). The cartridges used in this study were first washed with acetonitrile (5 ml) followed by washing with Milli-Q water (5 ml). Buffers (0.05 *M* sodium phosphate, pH 6.6 and 6.0) were prepared from 0.05 *M* disodium hydrogen phosphate solution adjusting pH to 6.6 or 6.0 with phosphoric acid.

2.2. Internal standard preparation

The internal standards, II and *p*-hydroxymethylbenzoate, were dissolved in a mixture of ethanol and water (1:1, v/v) and diluted to 80 $\mu g\ ml^{-1}$ and 20 $\mu g\ ml^{-1}$, respectively, with a mixture of ethanol and water (1:1, v/v) in a volumetric flask. The internal standard solutions were stable for more than one month at 4°C.

2.3. Apparatus and conditions

A LC-10A system liquid chromatograph equipped with a FCU-12AH column-switching device (Shimadzu, Kyoto, Japan) and a CCP&8000 series liquid chromatograph equipped with a PT-8000 column-switching device (Toso, Tokyo, Japan) and a L-4000 UV detector (Hitachi, Tokyo, Japan) were used. The detector wavelength was set at 210 nm. The samples were applied by a Rheodyne Model

7125 sample loop injector with an effective volume of 20 μl. HPLC was carried out on a 250×4.6 mm I.D. analytical column of L-column ODS (5 μm) (Chemical Inspection and Testing Institute, Tokyo, Japan) and 10×4 mm I.D. precolumn of Inertsil ODS-2 (5 μm) (GL Science, Tokyo, Japan) using acetonitrile–0.05 *M* sodium phosphate buffer (pH 6.6) (20:80, v/v) for assaying M1, M2 and M3 (solution A), acetonitrile–ethanol–0.05 *M* sodium phosphate buffer (32:6:62, v/v) (pH 6.6) for assaying I, M4, M5, M6 and M7 (solution B) and acetonitrile–0.05 *M* sodium phosphate buffer 1 (60:40, v/v) (pH 6.6) for cleaning precolumn (solution C). The flow-rate was 1.0 ml min^{-1} at 50°C. A schematic diagram of the column-switching system is shown in Fig. 2. This column-switching system was able to introduce the objective compounds into the analytical column and remove the late-eluted materials from the HPLC system.

In the case of M1, M2 and M3, the HPLC system had been previously equilibrated with solution A and a 20 μl sample solution was injected into the precolumn. After 1.3 min, the substances were introduced to the analytical column and the precolumn was washed with solution C 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.

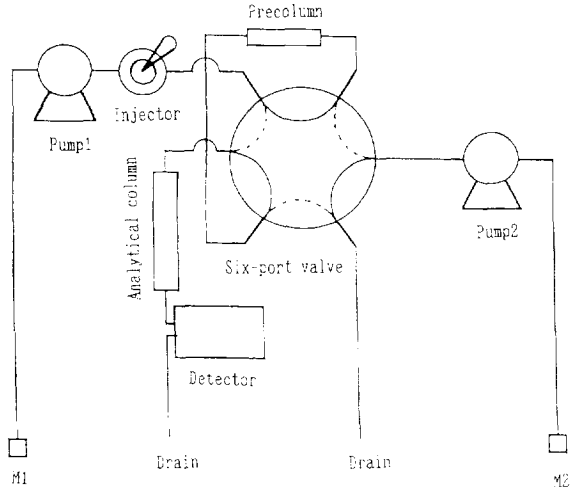


Fig. 2. Schematic diagram of column-switching system M1; solution A or solution B, M2; solution C. Solid line; equilibration and injection, dotted line; separation after column-switching time.

The assay of I, M4, M5, M6 and M7 was performed using the same procedures, except the switching time of introduction of substances to the analytical column was 1 min and the mobile phase was solution B.

2.4. Plasma collection

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

2.5. Sample preparation

A 0.05-ml volume of internal standard solution was spiked into 0.5 ml plasma in an eppendorf tube and then 0.5 ml of 0.05 *M* sodium phosphate buffer (pH 6.0) was added. The mixture was vortex-mixed for 10 s and applied to a Sep-Pak Vac tC_{18} cartridge which was preequilibrated with 5 ml of 0.05 *M* sodium phosphate buffer (pH 6.0). The cartridge was washed with 2 ml of water and finally 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 C and 20 μl of this solution was injected into HPLC system.

A 0.4-ml volume of internal standard solution and 1.4 ml of solution A were added to 0.2 ml of urinary sample in a test tube and the mixture was vortex-mixed for 10 s. An aliquot of 1 ml of the mixture was transferred to an eppendorf tube and centrifuged at 1000 *g* for 5 min. The supernatant was injected into the HPLC system.

3. Results and discussion

3.1. Sample preparation

The first effort was focused on establishing the preparation method of plasma and urinary samples by using SPE and dilution.

3.1.1. Plasma sample preparation

The preparation method of I in plasma which used Sep-Pak Light tC_{18} and the mixture of acetonitrile–

0.05 *M* sodium phosphate buffer (20:80, v/v) (pH 6.6) as washing solution [5] was applied to determination of metabolites in plasma, but the recoveries of metabolites were varied (10~93%), in particular the recoveries of M1, M2 and M3 were lower than 20%.

From the above results, both the SPE cartridge and the washing solution were investigated with many kinds of disposable cartridges being used to improve the recoveries of M1, M2 and M3. It was decided to use the Sep-Pak Vac tC_{18} as the SPE cartridge and water as a washing solution. The dilution mixture, 0.05 *M* sodium phosphate buffer (pH 6.0), was not changed and the eluent was ethanol.

3.1.2. Urinary sample preparation

The application of the preparation method for plasma samples enabled determination of the metabolites in urinary samples. In addition, the direct dilution method was tried as a simpler method. A comparison of SPE and the dilution method was made. It was found that for urinary sample preparation it was possible to replace the SPE method with the simple and rapid dilution method.

3.2. Chromatography

We previously developed [5] the assay method of I in plasma and urine by SPE using Sep-Pak Light tC_{18} and column-switching HPLC, which were equipped with L-column ODS column as an analytical column and Inertsil ODS-2 column as precolumn using acetonitrile–0.05 *M* sodium phosphate buffer (36:64, v/v) (pH 6.6) as mobile phase and this method was applied to assay the metabolites in plasma and urine. When the assays of blank samples spiked with the metabolites extracted from human urine were performed, the M1, M2 and M3 peaks overlapped in the front region and the M6 peak was not separated from that of the internal standard. The metabolites were classified into two groups according to their retention times, one group included M1, M2 and M3 which were eluted quickly and the other group was I, M4, M5, M6 and M7 which were eluted slowly.

The assay method of M1, M2 and M3 was examined by the variation of the amount of acetonitrile in mobile phase. Good separation of M1, M2 and M3 was achieved by changing the concentration of acetonitrile to 20% as shown in Fig. 3. It was decided to use *p*-hydroxymethylbenzoate as the internal standard because of separation of other substances and its retention time.

The effect on determination of I and M4, M5, M6 and M7 of adding ethanol or methanol to the eluent was examined. In previous examinations [4,5] to separate I from other substances in plasma, good separations were achieved using ethanol and metha-

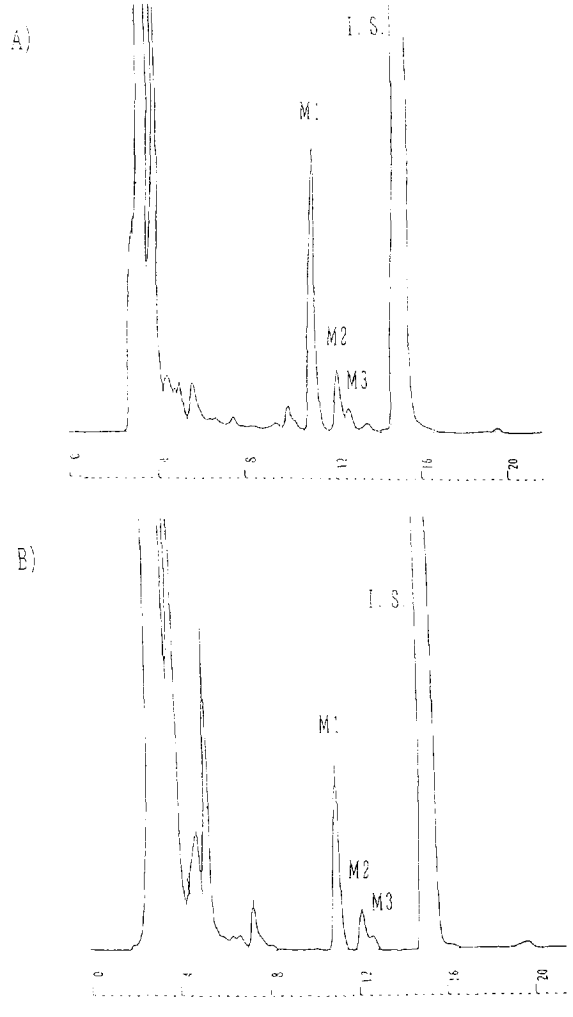


Fig. 3. Chromatograms of M1, M2 and M3 by column-switching HPLC with UV detection at 210 nm using solution A. (A) plasma sample spiked with I and its metabolites extracted from human urine, (B) urinary sample taken 0–4 h after oral administration of 60 mg I to human, I.S.; internal standard.

nol, as well as with acetonitrile. Since the addition of ethanol provided good separation as shown in Fig. 4, it was decided to use a mobile phase of acetonitrile–ethanol–0.05 *M* sodium phosphate buffer (32:6:62, v/v) (pH 6.6) and the internal standard II was used as in previous reports [4,5].

3.3. Determination of I and its metabolites

For the generation of calibration graphs, peak area ratios of M1, M2 and M3 to p-hydroxymethylbenzoate and I, M4, M5, M6 and M7 to II Were plotted against standard concentration. Over the concentration range 0.5–50 μg ml^{-1} of urine and 0.1–10 μg ml^{-1}, calibration graphs were found to be linear with correlation coefficients greater than 0.997. The recoveries of metabolites extracted from human urine were 88–100% and the R.S.D. values were 1–4% as shown in Table 1 and the recoveries of synthetic metabolites, M1, M2, M3 and M7, were 89–98% and the R.S.D. values were 3–6% as shown in Table 2.

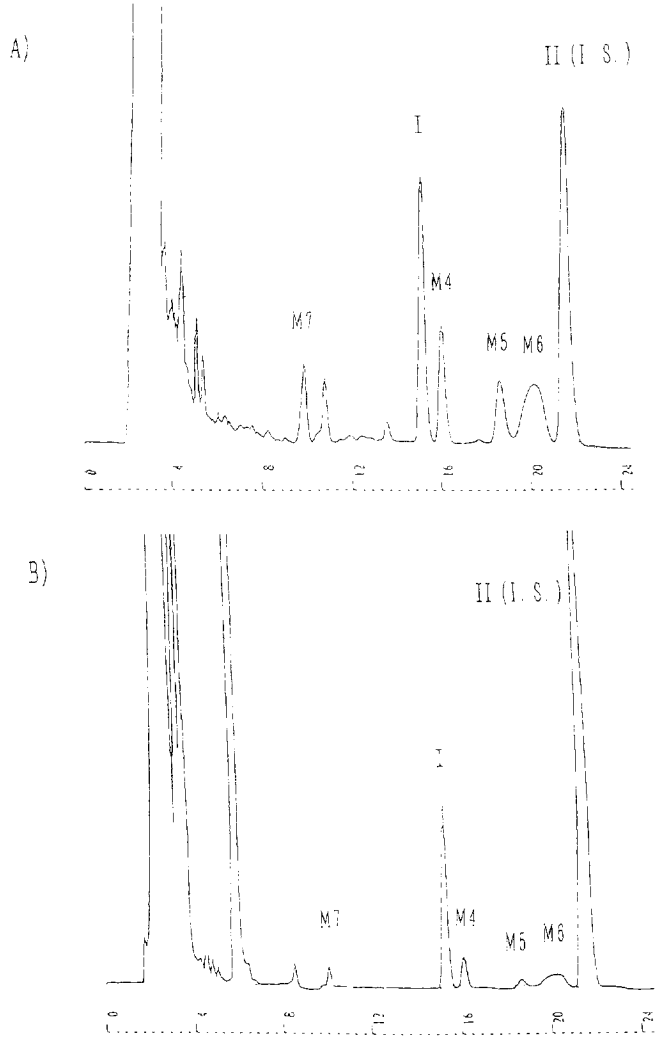


Fig. 4. Chromatograms of I, M4, M5, M6 and M7 by column-switching HPLC with UV detection at 210 nm using solution B. (A) plasma sample spiked with I and its metabolites extracted from the urine, (B) urinary sample taken 0–4 h after oral administration of 60 mg I to human, I.S.; internal standard.

Table 1
Analytical data for metabolites extracted from human urine spiked to plasma samples

Compound	Added ($\mu g\ ml^{-1}$)	Recovery (%)	R.S.D. (%)[a]
M1	41.5	99.9	3.0
M2	12.0	97.2	2.2
M3	4.0	97.2	2.8
I	11.6	96.9	0.8
M4	14.5	95.0	1.5
M5	3.1	94.2	4.0
M6	7.9	96.9	3.4
M7	3.1	88.2	1.1

[a] $n=7$.

Table 2
Analytical data for synthetic metabolites spiked to plasma samples

Compound	Added ($\mu g\ ml^{-1}$)	Recovery (%)	R.S.D. (%)[a]
M1	1.0	94.0	4.4
M2	1.0	95.2	4.9
M3	1.0	89.0	6.1
M4	1.0	98.2	3.2

[a] $n=5$.

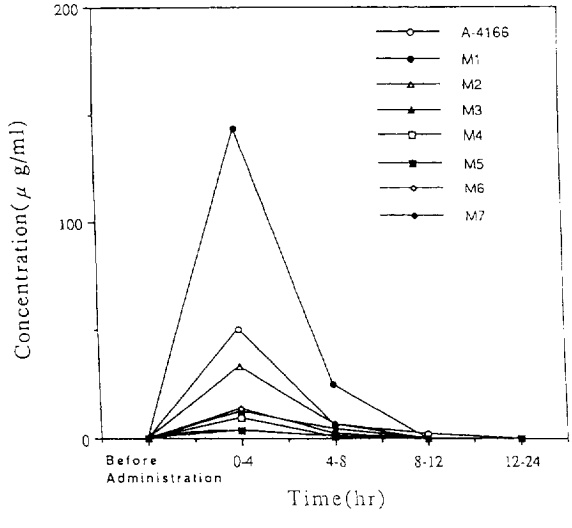


Fig. 5. Concentration–time curves for I and its metabolites (M1 to M7) in urinary sample after oral administration of 60 mg I to human.

When M4 was treated with β-glucuronidase, the peak area of M4 and I formed by deconjugation from M4 had much the same values. As M5 and M6 were presumed to have the same behavior as M4, it was decided that measured values of M4, M5 and M6 should be shown as converted from I values.

4. Application

Figs. 5 and 6 show the typical urinary and plasma concentration–time curves after administration of 60 mg I to humans. Seven metabolites were determined in urine but only two metabolites (M1 and M7) were determined in plasma. It was possible to measure even low concentrations of metabolites (M1 to 7) by the proposed methods.

5. Conclusion

Seven metabolites were found to be present in human plasma and/or urine. Measurement of these metabolites were carried out by two column-switching HPLC conditions, because metabolites were classified into two groups according to their different retention times. In the present study, two column-switching HPLC methods with UV detection at 210 nm were investigated. SPE method was useful for the sample preparation of plasma and the direct

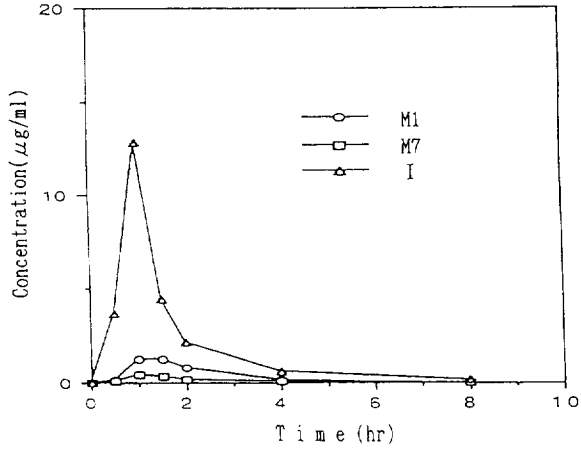


Fig. 6. Concentration–time curves for M1 and M7 in plasma sample after oral administration of 60 mg; I to human.

dilution method was useful for urinary samples. This proposed method established here is applicable to simultaneous determination of low levels of I and its metabolites in human plasma and urine.

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