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Short Communication

Determination of a new calcium antagonist and its main metabolite in plasma by thermospray liquid chromatography–mass spectrometry

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ABSTRACT

A highly sensitive thermospray liquid chromatographic–mass spectrometric method has been developed for the simultaneous determination of FRC-8653 (I), a new calcium antagonist, and its main metabolite (M-4) in plasma. A deuterated analogue of I was added to the plasma as the internal standard. After the purification and concentration of the plasma sample on bonded-phase disposable columns, the extract was injected into the thermospray liquid chromatograph and analysed by selected-ion monitoring mass spectrometry. The calibration curves obtained were linear over the concentration range 0.5–100 ng/ml. The limits of quantification are 0.5 ng/ml for I and 1 ng/ml for M-4 in plasma, which are sufficient to evaluate plasma concentrations after oral administration to rats.

INTRODUCTION

FRC-8653 [2-methoxyethyl-(*E*)-3-phenyl-2-propen-1-yl-(±)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate, I, see Fig. 3A] is a new dihydropyridine derivative with calcium antagonist activity [1]. The drug, originally found by Fujirebio (Tokyo, Japan), has been developed in collaboration by Ajinomoto and Fujirebio, and is undergoing clinical evaluation for antihypertensive effect. Pharmacokinetic studies of the administration of I required a sensitive assay method capable of quantifying concentrations of 1 ng/ml or less for I and its main metabolite (M-4) in plasma.

Gas chromatography (GC) [2–4] and gas chromatography–mass spectrometry (GC–MS) [5–10] are used for the determination of many other dihydropyridine derivatives in biological fluids. However, these methods were not suitable for I, because it decomposes under GC conditions. Furthermore, liquid chromatography (LC) with UV detection was not successful for detailed pharmacokinetic studies, because of the presence of interfering peaks in chromatograms obtained from blank plasma samples.

This paper describes a sensitive and selective thermospray LC–MS method, applicable to

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pharmacokinetic studies, for the quantification of I and its main metabolite in plasma.

EXPERIMENTAL

Reagents and materials

Compound I and its deuterium-labelled compound (d_3-I, see Fig. 3C) were synthesized and supplied by Fine Chemicals Development Laboratories in our laboratories. The main metabolite of I (M-4, see Fig. 3B) was obtained from Fujirebio. Acetonitrile was of HPLC grade from Wako (Osaka, Japan), and the other reagents were of analytical grade. Bond-Elut C_{18} columns (No. 607203) were obtained commercially from Uniflex (Tokyo, Japan). The internal standard was dissolved in acetonitrile and diluted to 10 μg/ml with acetonitrile.

Liquid chromatography

The LC system consisted of an L-6200 pump from Hitachi (Tokyo, Japan), a Rheodyne 7125 (20-μl loop) injector, and Hypersil ODS columns (60 mm × 4.6 mm I.D.) from Hewlett-Packard (Palo Alto, CA, USA), packed with 3-μm particles. The mobile phase was acetonitrile–0.1 *M* ammonium acetate (60:40) at natural pH, filtered through a 0.45-μm filter.

The system was operated at a flow-rate of 1 ml/min, and the column was kept at room temperature.

Mass spectrometry

A Hewlett-Packard thermospray LC–MS Model 5988A quadrupole mass spectrometer was employed. The working temperatures were 95°C for the vaporizer stem and 275°C for the ion source. The mass spectrometer was operated in

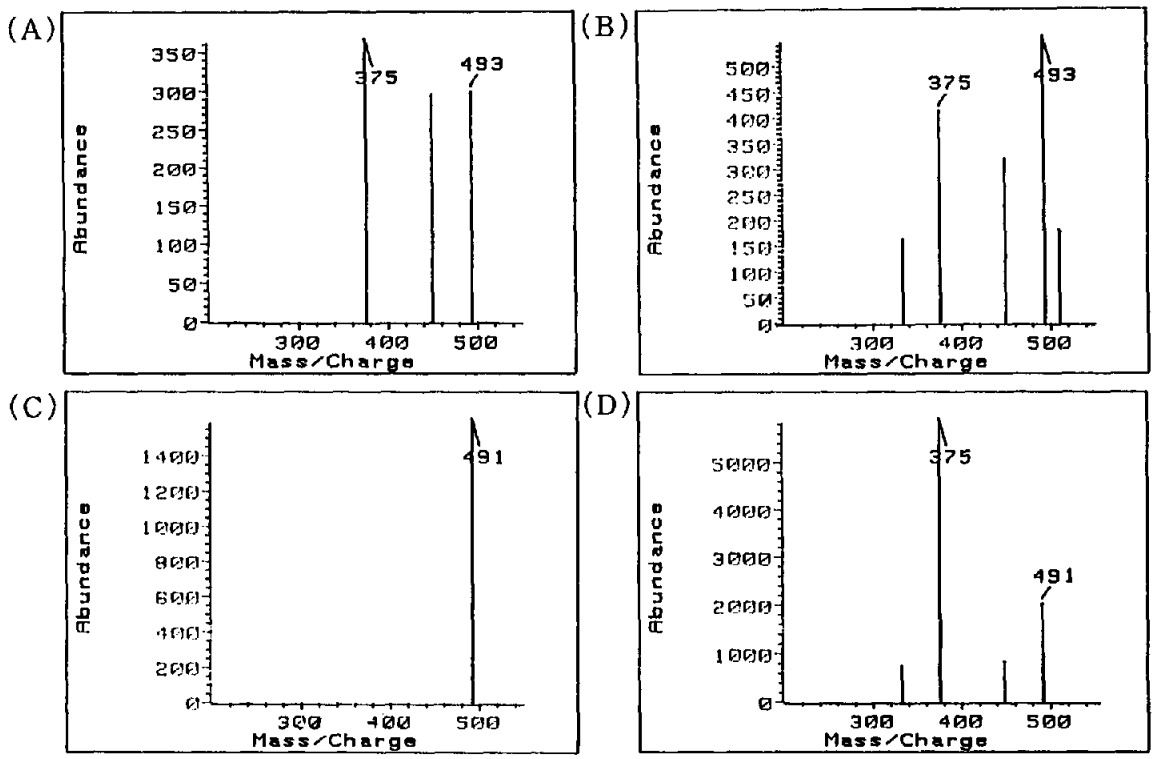

Fig. 1. Mass spectra for I obtained in four analytical modes. (A) Positive-ion filament OFF mode; (B) positive-ion filament ON mode; (C) negative-ion filament OFF mode; (D) negative-ion filament ON mode.

negative-ion filament ON mode. Spectra were acquired from m/z 200 to 550 at a rate of 2 s per scan. For selected-ion monitoring (SIM) measurements, the monitoring ions were set to m/z 375 for I, m/z 378 for d_3-I and m/z 361 for M-4, and scanned at 0.1 s per ion (0.3 s total scan time).

Extraction procedure

The plasma samples were stored at −20°C until analysis.

A 100-ng amount of d_3-I was spiked into 1 ml of plasma sample in a centrifuge tube, and then 2 ml of acetonitrile were added. The mixture was vortex-mixed for 30 s. After centrifugation at 1670 *g* at 4°C for 10 min, the supernatant was transferred to another centrifuge tube using a disposable Pasteur pipette. The extraction was repeated with a further 1 ml of acetonitrile.

Then 6 ml of water were added to the second tube and the contents were vortex-mixed for 30 s. The mixture was applied to a Bond Elut C_{18} column, which was pretreated with 5 ml of acetonitrile then 10 ml of water. The column was washed with 2 ml of acetonitrile–water (4:6, v/v) and finally eluted with 2 ml of acetonitrile. The eluate was evaporated to dryness with a rotary evaporator at 40°C. The residue was dissolved in 100 μl of acetonitrile, and 10 μl of the solution was injected into the LC–MS system.

RESULTS AND DISCUSSION

Modes of ionization

A 100-ng sample of I was analysed in all four different modes of operation (positive-ion filament OFF mode, positive-ion filament ON mode, negative-ion filament OFF mode and negative-ion filament ON mode). These data were then compared with respect to the sensitivity, fragmentation and molecular mass information (Fig. 1).

In the positive-ion modes the fragments $[M + H]^+$ (m/z 493) and $[M + NH_4]^+$ (m/z 510) were observed, with a few fragment ions corresponding to the loss of the side-chain. The total-ion current sensitivity in the positive-ion filament ON mode was approximately double that in the positive-ion filament OFF mode.

In the negative-ion filament OFF mode, the mass spectrum was simple, and only the $[M - H]^-$ ion was observed. In the negative-ion filament ON mode, the fragmentation and sensitivity were enhanced. The total ion current sensitivity in the latter mode was approximately one order of magnitude higher than that in the positive-ion filament ON mode; this may be caused by the presence of the NO_2 group, which has a high electron affinity [6,11]. Thus the negative-ion filament ON mode was selected for further study.

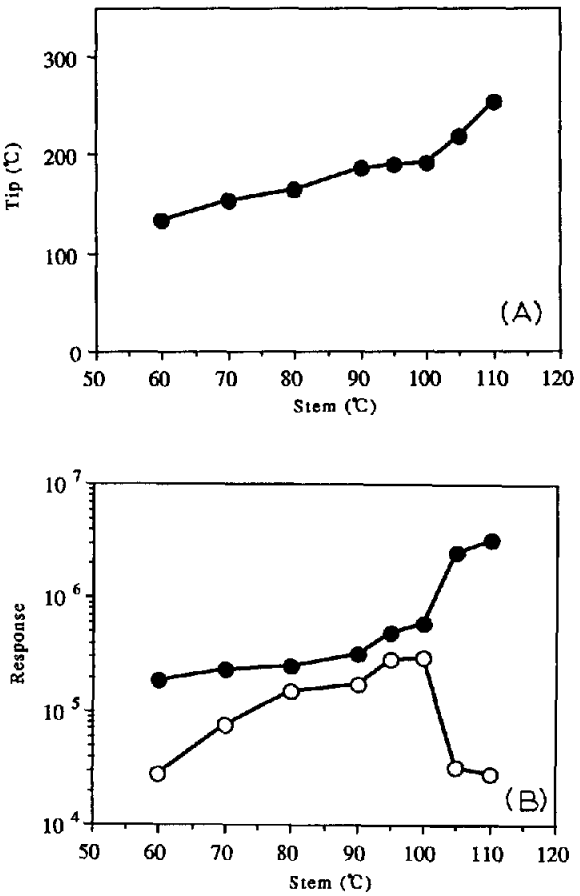

Fig. 2. Effect of vaporizer temperature on thermospray sensitivity. (A) Vaporizer tip temperature as a function of vaporizer stem temperature for a mobile phase flow-rate of 1 ml/min; (B) response of I as a function of stem temperature; (○) deprotonated molecular ion (m/z 491); (●) main fragment ion (m/z 375) of I.

Optimization of vaporizer temperature

Two thermocouples were used in the vaporizer for the optimization of the MS system. One was located near the inlet end of the probe and controlled the vaporizer temperature. The other was near the tip of the vaporizer [12,13].

Fig. 2A shows the vaporizer tip temperature as a function of vaporizer control temperature (stem temperature) when the flow-rate of the mobile phase through the vaporizer capillary was 1 ml/min. This graph indicates that complete vaporization occurred at *ca.* 100°C (take-off temperature). The profiles of the responses of the parent ion and the main fragment ion *versus* stem temperature are shown in Fig. 2B. Increasing temperature afforded higher ion intensity up to take-off temperature. Above the take-off temperature, the parent ion response fell in a logarithmic fashion, whereas the main fragment ion response increased dramatically. For sensitive

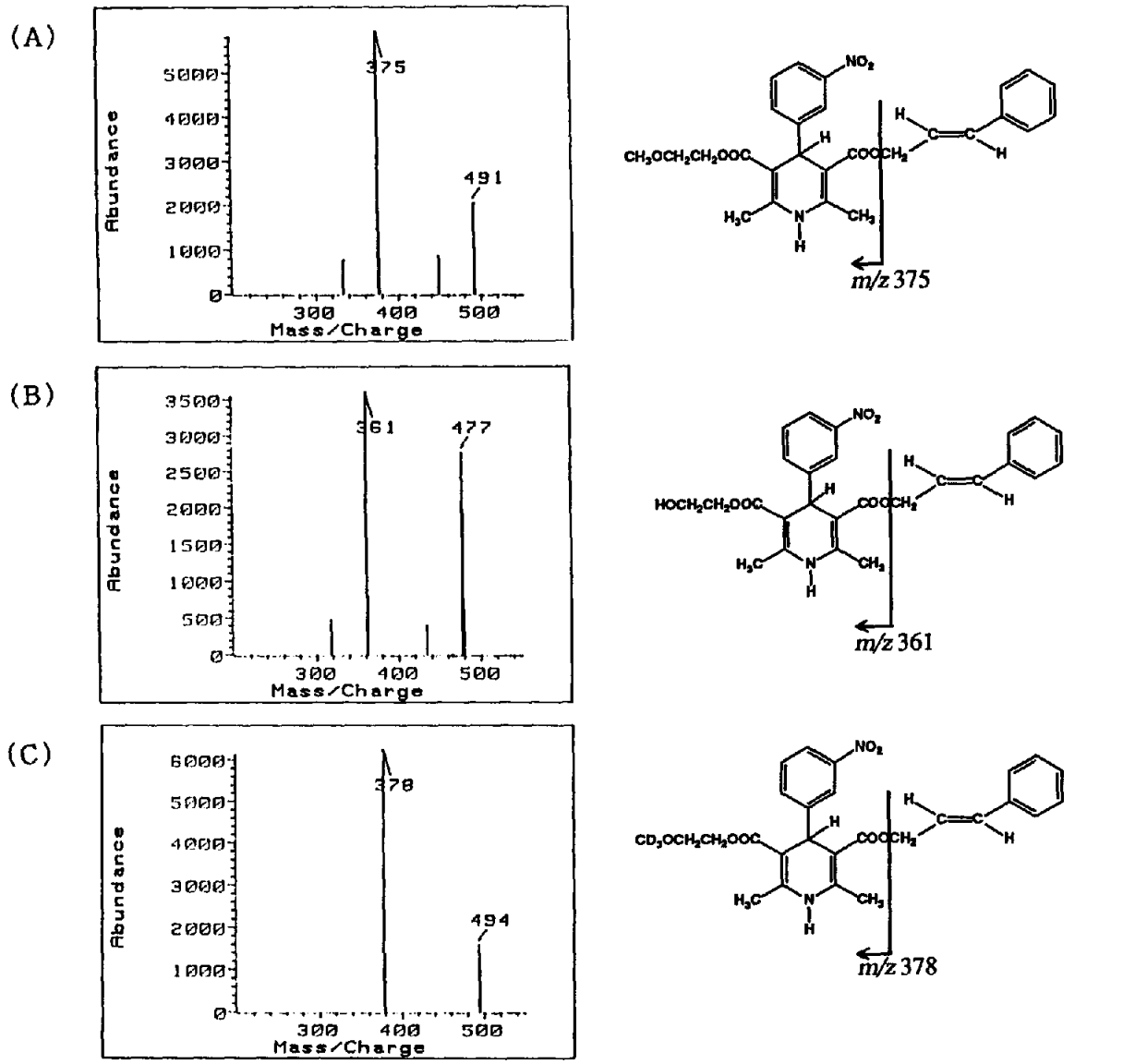

Fig. 3. Mass spectra of I (A), M-4 (B), and d_3-I (C) under the optimum condition (negative-ion filament ON mode). The specific fragments corresponding to the indicated ions are depicted on the structures.

analysis, the stem temperature was set at 110°C, but prolonged operation under these conditions led to sample decomposition and plugging of the vaporizer capillary. Thus the stem temperature was set at 95°C, *ca.* 95% of the vaporization temperature, for the optimum combination of sensitivity and longevity of the vaporizer [14,15].

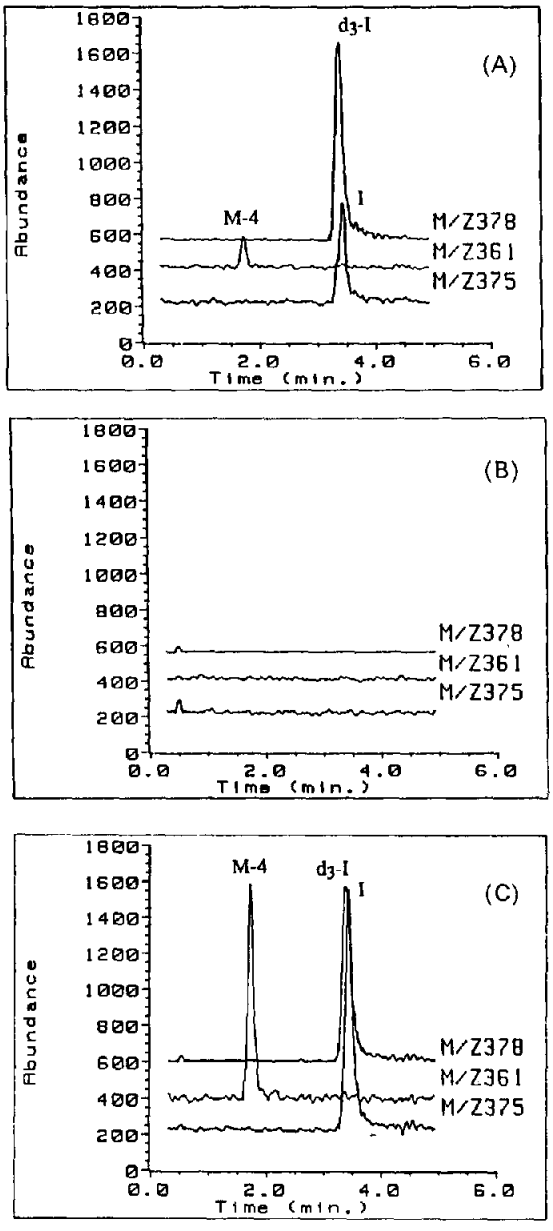

Fig. 4. SIM chromatograms obtained by monitoring ions at *m/z* 375, 361 and 378 for I, M-4 and d_3-I, respectively. (A) Standard solution containing 500 pg of I, 500 pg of M-4 and 10 ng of d_3-I (1:10 scale); (B) blank rat plasma; (C) plasma sample taken 6 h after oral administration of 10 mg/kg I to a male rat.

Specificity of thermospray LC–MS method

Mass spectra of I, M-4, and d_3-I, obtained using thermospray LC–MS in the negative-ion filament ON mode, are shown in Fig. 3. With regard to the sensitivity and specificity, the optimal monitoring ions were selected at *m/z* 375, 361 and 378 for I, M-4 and d_3-I, respectively. Typical SIM chromatograms obtained from the standard solution, blank rat plasma, and a plasma sample taken 6 h after oral administration of 10 mg/kg I to a male rat are shown in Fig. 4. The SIM chromatogram of the blank rat plasma (Fig. 4B) shows no interference from co-eluting components derived from plasma at the retention times of I, M-4, and d_3-I, which demonstrates the high specificity of this method. The 6-h plasma sample showed a response equivalent to the concentrations of 15.6 ng/ml for I and 45.0 ng/ml for M-4 (Fig. 4C).

Linearity and quantification limit

For the generation of standard curves, peak-height ratios of I and M-4 to d_3-I were plotted against standard concentration. Over the concentration range 0.5–100 ng/ml, the standard curves

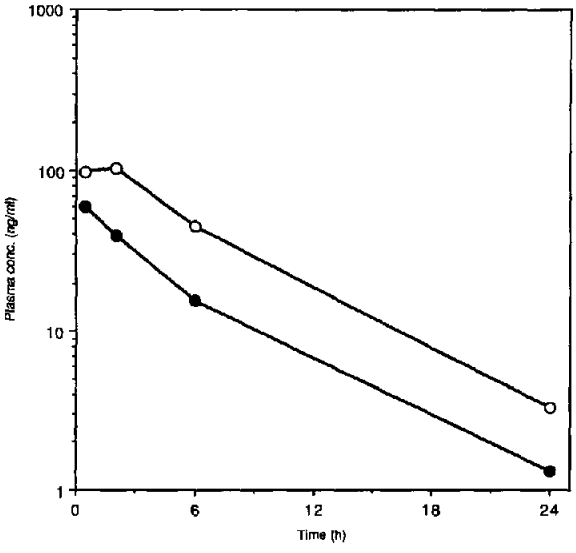

Fig. 5. Concentration–time curves for I (●) and M-4 (○) after oral administration of 10 mg/kg I to a male rat.

TABLE I

REPRODUCIBILITY OF DETERMINATION FOR I AND M-4 IN RAT PLASMA

Compound	Actual concentration (ng/ml)	Mean concentration found[a] (ng/ml)	Percentage of actual concentration	Range (ng/ml)	S.D. (ng/ml)	R.S.D. (%)
I	2.00	1.89	94.3	1.73–2.03	0.123	6.5
	10.0	9.36	93.6	8.79–9.75	0.346	3.7
M-4	10.0	9.43	94.3	8.33–10.0	0.698	7.4

[a] $n = 5$.

were found to be linear with correlation coefficients greater than 0.997. The quantification limit of this method is 0.5 ng/ml for I and 1 ng/ml for M-4 in plasma.

Reproducibility

The reproducibility was determined by performing five replicate analyses of spiked rat plasma samples. The results are given in Table I. This method provided good accuracy and precision.

Application

Fig. 5 shows typical plasma concentration–time curves after oral administration of 10 mg/kg I to a male rat. It was possible to measure even low concentrations of I and M-4 with this method, which is appropriate for pharmacokinetic studies of I in rats.

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