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Short communication

Identification of YH439 and its metabolites in rat urine by gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry

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Abstract

YH439 is a potential drug candidate for the treatment of various hepatic disorders. YH439 and its three metabolites have been identified in rat urine by liquid chromatography–mass spectrometry (LC–MS) and by gas chromatography (GC)–MS. Identification of YH439 and its metabolites was established by comparing their GC retention times and mass spectra with those of the synthesized authentic standards. Both electron impact- and positive chemical ionization MS have been evaluated. The metabolism study was performed in the rat using oral administration of the drug. A major metabolite (YH438) was identified as the N-dealkylation product of YH439. Other identified metabolites were caused by the loss of the methyl thiazolyl amine group (metabolite II) from YH439, the isopropyl hydrogen malonate group (metabolite IV) and the decarboxylated product (metabolite III) of metabolite II. © 1997 Elsevier Science B.V.

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1. Introduction

YH439 is a novel agent that can be used clinically for the improvement and treatment of symptoms of various hepatic disorders, including hepatitis, fatty liver, fibrosis and chemical intoxication caused by alcohol and other chemicals. The chemical structure of YH439, isopropyl 2-(1,3-dithiethan-2-ylidene)-2-[N-(4-methylthiazol-2-yl) carbamoyl] acetate, is shown in Fig. 1. The lead compound of YH439 is malotilate(diisopropyl 1,3-dithiol-2-ylidene malo-

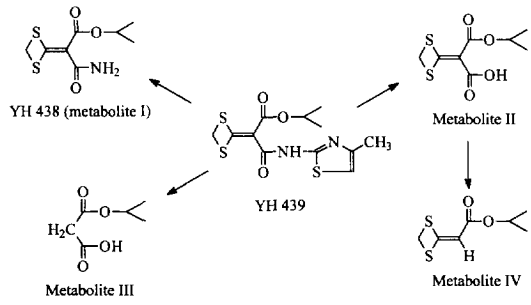

Fig. 1. Chemical structure of YH439 and its metabolites.

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nate), which was discovered by the Nihon Nohyaku Company in Japan [1]. Over 100 dithiolane derivatives were synthesized and tested for their pharmacological action in improving liver function. Studies on the metabolism of malotilate were performed [2–6]. YH439, which is currently under phase I clinical trials as an hepatoprotective agent in Germany, has been proved, by a series of animal studies, to be potent and very safe. A metabolism study was done in rats to find its metabolites in biological fluids. Three different metabolites, as well as the parent compound (Fig. 1), were identified in rat urine and the chemical structures of these were determined using authentic compounds synthesized at the Yuhan Research Center.

2. Experimental

2.1. Reagents and material

YH439 and its authentic metabolites were obtained from Yuhan Research Center (Ahn Yang, Korea). Methyl iodide and *o*-(4-nitrobenzyl)-N,N′-diisopropylisourea (NBDI) were purchased from Sigma (St. Louis, MO, USA). All reagents were of analytical grade.

2.2. Gas chromatography–mass spectrometry (GC–MS)

GC–MS analyses were performed with a HP 5988A mass spectrometer (Hewlett-Packard, CA, USA) coupled to a HP 5890A gas chromatograph (Hewlett-Packard). The mass spectrometer was operated with a filament current of 300 μA and an electron energy of 70 eV in the electron impact (EI) mode and at 230 eV in the chemical ionization (CI) mode. Methane reagent gas for CI MS was introduced into the ion source through a transfer line after passing it through a trap of activated charcoal and a molecular sieve. The ion source was held at 200°C and at 106 Pa in the CI mode. A cross-linked methylsilicon capillary (12 m×0.3 mm I.D.; film thickness, 0.33 μm) column was installed in the gas chromatograph and inserted directly into the ion source of the mass spectrometer. Helium (0.9 ml/min) was used as the carrier gas. The oven was held at 100°C for 0.5 min and the temperature was increased to 300°C at a rate of 20°C/min and finally it was held at 300°C for 3 min. The split ratio was 1:10. The temperature of the injector and transfer line were maintained at 290°C.

2.3. Liquid chromatography–mass spectrometry (LC–MS)

A thermospray interface (HP 5985-7598, Hewlett-Packard) was used for introducing effluent from the high-performance liquid chromatography (HPLC) system (HP 1090A, Hewlett-Packard) into a mass spectrometer (HP 5988A, Hewlett-Packard). The column was a Hypersil ODS column (100×4.6 mm I.D., 5 μm, Hewlett Packard). The mobile phase consisted of solvent A (methanol) and solvent B (ammonium acetate buffer). A linear gradient (0.8 ml/min) was programmed from 10 to 70% A in 30 min.

2.4. HPLC system

A Young-In 910 solvent delivery system (Young-In, Korea) was used with a μ-Bondapack C_{18} column (30 cm×3.9 mm I.D., 10 μm, Waters), a Rheodyne 7725 sample injector and a Young-In M-720 UV–Vis variable detector (Young-In). The mobile phase consisted of acetonitrile–water–acetic acid (50:49:1, v/v/v).

2.5. Metabolic study in rats

Male Sprague-Dawley rats, weighing ca. 250 g, were housed in metabolic cages. Urine was collected for 24 h before the oral administration. YH439 was suspended in a 0.5% CMS (carboxymethyl cellulose) solution and was given at a dose of 200 mg/kg. After dosing, urine was collected for 24 h and stored at −20°C until analysis.

2.6. Extraction and separation

A 10-ml volume of urine was added to an Amberlite XAD-2 column (100–200 μm). The column (pasteur pipette, closed with glass pearl, bed height 2.5 cm) was washed with 2 ml of distilled

water, and the absorbed fraction was eluted four times with 1 ml of methanol. The methanolic eluate was evaporated to dryness using a vacuum rotary evaporator. The residue was dissolved in 1 ml of water, and 5 ml of diethyl ether were added to the aqueous layer. After shaking for 5 min and centrifugation (2000 rpm), the ether layer was transferred to another tube and evaporated to dryness. The residue was dissolved in 500 μl of methanol prior to GC–MS injection.

2.7. Methyl derivatization ([7])

The dried methanol residue was mixed with 180 μl of acetone, 20 μl of methyliodide and 100 mg of potassium carbonate. The reaction mixture was heated at 70°C for 2 h and injected into the GC–MS system after being cooled.

2.8. Identification of metabolite IV (isopropyl malonic acid) (3)

A mixture containing 1 ml of urine sample, 0.5 ml of 1 *M* HCl and 5 ml of ethylacetate was shaken for 5 min and centrifuged. The organic phase was transferred to a centrifuge tube. The ethyl acetate phase was dried with 2 g of sodium sulfate. After centrifugation for 5 min, 4 ml of the ethyl acetate extract were placed in a new tube. Then 1 ml of NBDI (dissolved in a methylene chloride solution) was added and evaporated under a stream of nitrogen at 60°C to a volume of ca. 0.5 ml. The reaction mixture was heated at 70°C for 2 h, and the solvent was evaporated to dryness under a gentle stream of nitrogen. In the case of HPLC, the residue was dissolved in 0.6 ml of *n*-hexane and 1 ml of 0.01 *M* HCl was added. A 20-μl volume of organic phase was injected into the HPLC system. The column was μ-Bondapak C_{18} (300×3.9 mm) and the UV detector system was operated at 269 nm. The mobile phase consisted of methanol–water–acetic acid (45:50:5, v/v/v). The flow-rate was set at 1 ml/min. The retention time was 18.14 min. In the case of GC–MS, the sample that eluted at 18.14 min from the HPLC system was dried and dissolved in *n*-hexane prior to injection into the GC–MS system.

3. Results and discussion

Three metabolites were identified from the urine sample of a rat that had been treated orally with YH439 suspended in 0.5% CMC solution.

3.1. YH439

YH439 was detected at 10.79 min using the EI–GC–MS system (Fig. 2). The mass spectrum of the extracted YH439 was identical to that of authentic YH439. Because the carboxylic acid group is absent, the mass spectrum of YH439 is identical to both the pre- and post-derivatization mass spectra. The mass spectrum obtained from positive chemical ionization (PCI) MS showed a pseudo-molecular ion of *m/z* 331 $[M+H]^+$ and other ions at *m/z* 359 $[M+C_2H_5]^+$. Therefore, the separated compound is YH439.

3.2. Metabolite I (YH438)

Metabolite I, so called YH438, is an N-dealkylated product and was detected in urine by GC–MS. Metabolite I was detected at a retention time of 6.52 min (Fig. 2). Both the retention time and the mass spectra of the synthesized authentic YH438 were identical to those of the extracted YH438 in which the 4-methyl thiazolyl group is lost. Under EI mass spectrometric conditions, the molecular ion of this metabolite was observed at *m/z* 233, indicating the elimination of the methylthiazolyl ring from the YH439. In addition, the ions $[M-OCH(CH_3)_2]^+$ and $[M-COOCH(CH_3)_2]^+$ were observed at *m/z* 174 and 146, respectively. The mass spectrum obtained from PCI MS, with methane as the reactant gas, shows a pseudo-molecular ion of *m/z* 234 $[M+H]^+$ and other ions of *m/z* 262 $[M+C_2H_5]^+$. Thus, the metabolite was identified as a YH438.

3.3. Metabolite II

Under the EI–GC–MS conditions used, metabolite II was not detected because of its highly polar carboxylic group. The mass spectrum obtained from LC–MS showed pseudo-molecular ions of *m/z* 235 $[M+H]^+$ and *m/z* 252 $[M+NH_4]^+$ (Fig. 3). Hence, the molecular mass of this metabolite was assumed

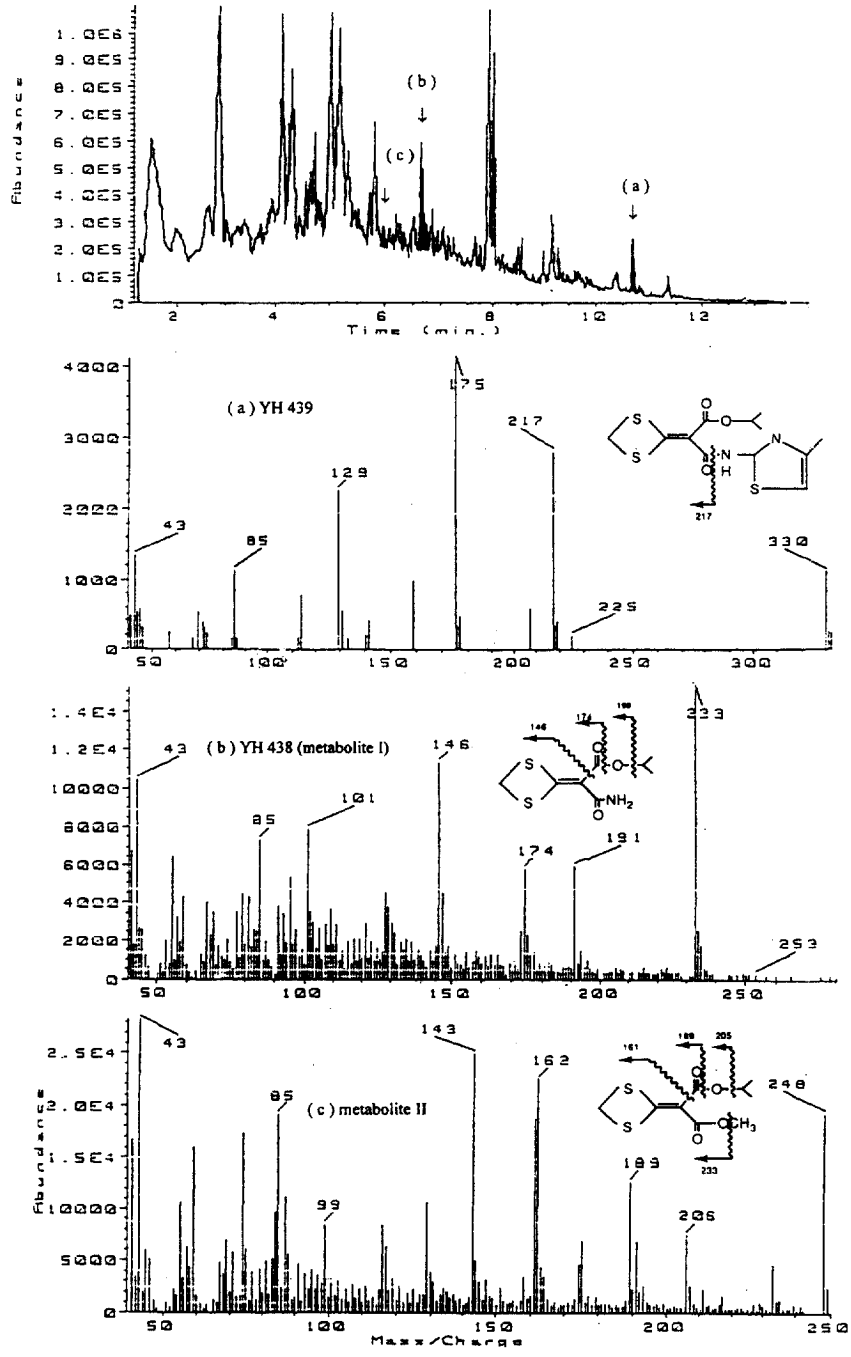

Fig. 2. Total ion chromatogram (top) and GC–MS spectra of the metabolites isolated from rat urine extracts; (a) YH439, (b) YH438 (metabolite II), (c) metabolite III.

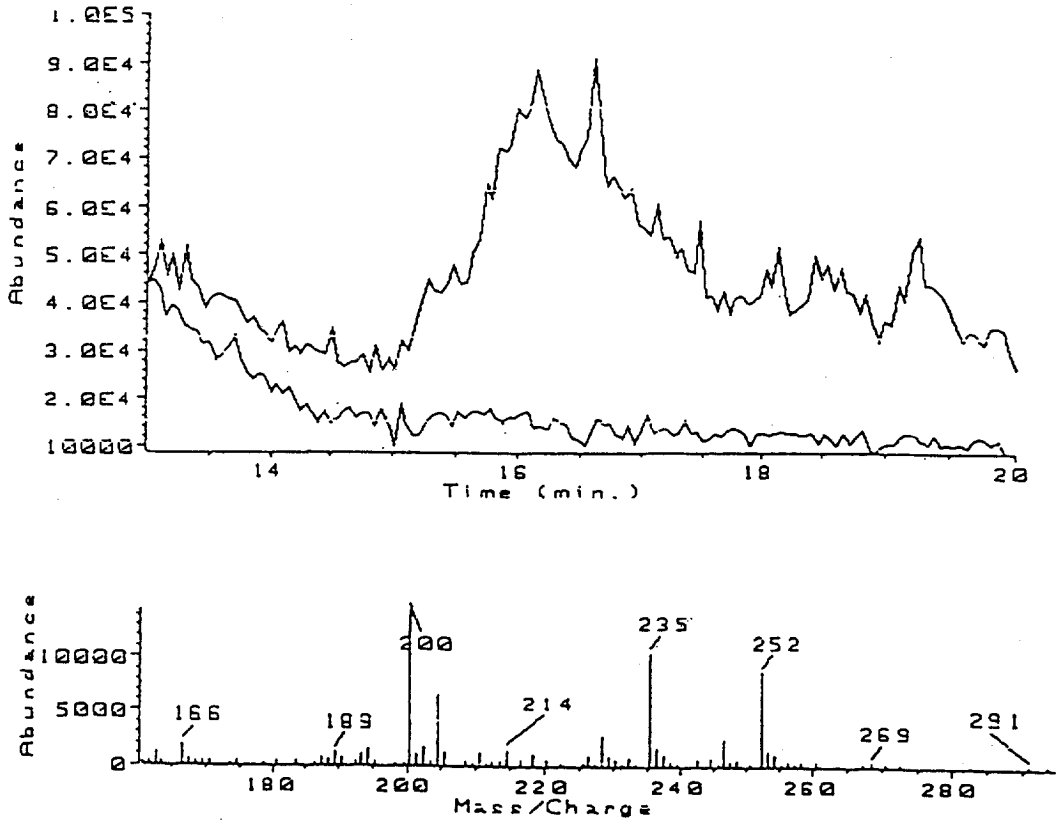

Fig. 3. LC–MS chromatogram and mass spectrum of metabolite III isolated from rat urine.

to be 234. The methyl derivative of this metabolite was detected at 5.91 min under the EI–GC–MS conditions used. The molecular ion of its methyl derivative was observed at *m/z* 248. The ions of *m/z* 189 and 162 were $[M-OCH_2(CH_3)_2]^+$ and $[M-COOCH_2(CH_3)_2]^+$, respectively. From PCI–GC–MS, the pseudo-molecular ion $[M+H]^+$ was detected at *m/z* 249 and that of $[M+C_2H_5]^+$ was detected at *m/z* 277. Both the retention time and the mass spectra of the synthesized authentic standard were identical to those of the detected metabolite. Hence, metabolite II, a molecular ion of *m/z* 234 with a functional carboxylic acid group, was identified as a metabolite of YH439.

3.4. Metabolite III

The compound thought to be metabolite III, detected at 3.65 min, was, in fact, a thermal degradation product of metabolite II, generated in the injection port of the GC system. As evidence, after methylation, metabolite III was not detected at 3.65 min, while synthesized metabolite II without derivatization was detected at 3.65 min on GC–MS. The characteristic ions of the degradation product were detected at *m/z* 190 $[M]^+$, 131, 103 and 85. Thus, metabolite III is not a true metabolite of YH439.

3.5. Metabolite IV

By comparing the HPLC chromatogram of the NBDI derivative of the blank urine and of the urine sample following administration of drug, metabolite IV was identified as isopropyl hydrogen malonate. Its NBDI derivative was detected at a retention time of 8.59 min, under EI–GC–MS conditions. The retention time and mass spectrum were identical to

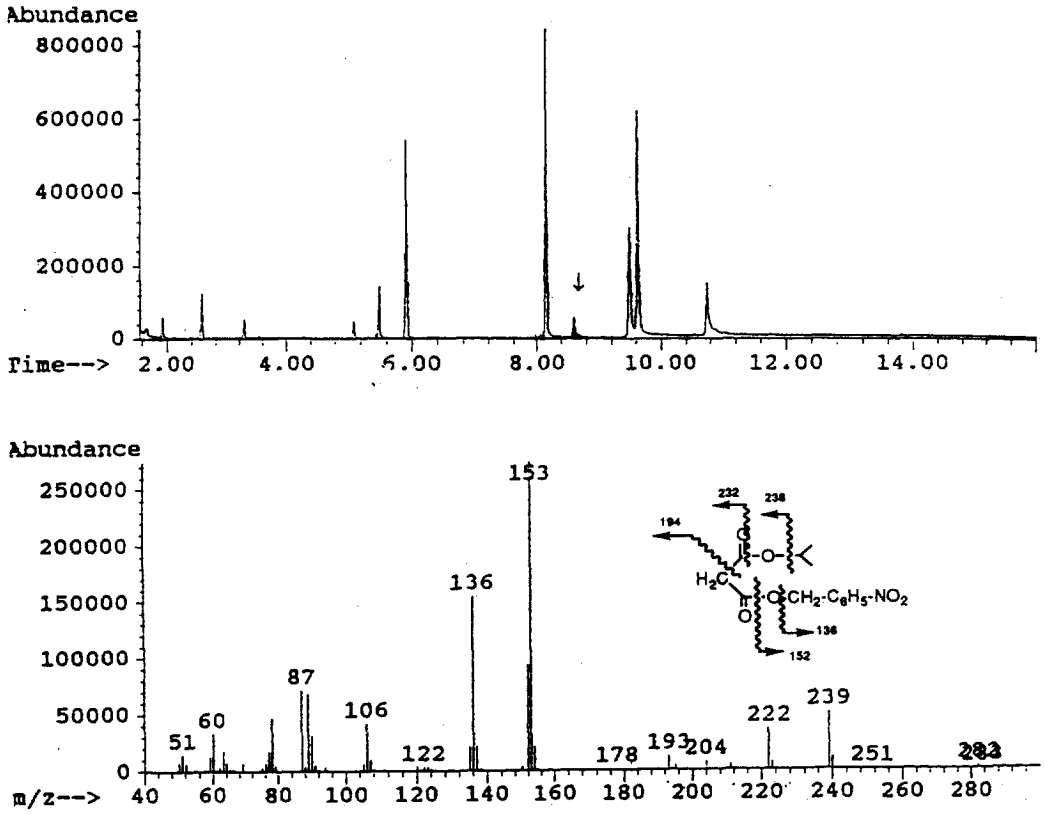

Fig. 4. Total uin chromatogram and mass spectrum of derivatized metabolite IV with NBDI, of a sample isolated from rat urine.

those of the synthesized authentic standard, both showing characteristic ions at *m/z* 239, 222, 153 and 136 (Fig. 4).

4. Conclusion

The identification of YH439 metabolites in rat urine was established by comparing their GC retention times and their electron impact and methane chemical ionization mass spectra. The three metabolites consisted of the N-dealkylation product of YH439 (YH438), metabolite II, which was caused by the loss of the methyl thiazolyl amine group from YH439, isopropyl hydrogen malonate (metabolite IV) and the decarboxylated product (metabolite III) of metabolite II.

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