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Pharmacological Properties of Galenical Preparation (XIII) Metabolites in Rat Urine of Orally Administered Evodia Fruits (呉茱萸) Extract

YOSHIHIRO KANO,* SATORU KANEMAKI, MASAHO YASUDA, KEN-ICHI SAITO and KEN-ICHI KOMATSU

Hokkaido Institute of Pharmaceutical Sciences, 7–1 Katuraoka-cho, Otaru 047–02, Japan

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After the oral administration of a methanol extract of "Evodia Fruit (呉茱萸)," rat urine was examined for its metabolites by using HPLC equipped with a fluorescence detector. The 4-quinolone type compound 5-(1, 4-dihydro-1-methyl-4-oxo-2-quinolin-2-yl)-pentanoic acid was detected as the main metabolite in the urine, and it was seemed to be derived from evocarpine which was contained in the crude drug, Evodia Fruit.

Keywords——*Evodia officinalis*; Rutaceae; Evodia Fruit; 5-(1, 4-dihydro-1-methyl-4-oxo-2-quinoline-2-yl)-pentanoic acid; 4-quinolone; metabolite in rat urine; HPLC, NMR

"Evodia Fruit (Goshuyu, 呉茱萸)" included in JP XI is prepared from fruits of *Evodia rutaecarpa* BENTHAM or *E. Officinalis* DODE (Rutaceae), and is used in some traditional Chinese prescriptions, for example "Goshuyu-to (呉茱萸湯)" and "Toki-shigyaku-ka-goshuyu-shokyo-to (当帰四逆加呉茱萸生姜湯)." Evodia Fruit is said to be effective in improving the low metabolic state induced by general prostration (虚) following a chronic disease. The symptoms said to be effectively treated with it are chilled limbs, migraines, stomachache and nausea.

As the main chemical compounds of Evodia Fruit, indolquinazoline alkaloid evodiamine,[1] rutaecarpine[1] and hydroxyevodiamine,[2] 4-quinolone alkaloid evocarpine[3] and bitter compound limonin[4] are known and many pharmacological studies on these compounds have been made.[5] In this study, a methanol extract of the crude drug, Evodia Fruit was orally administered to rats, and the rat urine was analyzed. 5-(1, 4-Dihydro-1-methyl-4-oxo-2-quinolin-2-yl)-pentanoic acid having a 4-quinolone structure was the major metabolite detected in the urine.

Experimental

Plant material——"Evodia Fruit (呉茱萸)" that conformed to the description of JP XI were commercially obtained in Osaka in 1987, which was identified as the fruit of *Evodia officinalis* by the external characteristics.[6]

Experimental animals——Male Wistar rats, six weeks of age, obtained from Shizuoka Laboratory Animal Center, Hamamatsu, Japan were used. The animals were kept at 24 ± 1°C with a 12 h dark-light cycle.

Analysis of urine——Administration: 50g of Evodia Fruit was extracted twice with 500 ml of boiling methanol. The combined solutions were concentrated in vacuum. Then the extract was diluted with water containing 2% Tween 80 to prepare solutions which contained 1.0 g/ml, 2.0 g/ml and 3.0 g/ml equivalent of the crude drug. 0.5 ml of sample solution was orally administered to a rat.

Urine samples: Rat urine was collected for 24 h after the oral administration of the drug by using a metabolic cage (daily rat urine was about 8 ml). The urine was diluted 5 times with methanol, filtered through a 0.45 μm filter and used for the HPLC analysis (Fig. 1-B).

In order to identify the chemical compounds in the crude drug Evodia Fruit, the methanol extract was first analyzed by HPLC (Fig. 1-C). This HPLC sample solution was prepared by adding 8 ml (daily amount of rat urine) of methanol to 0.5 ml of 3.0 g/ml extract (in conversion into crude drug), then diluting the solution 5 times with methanol and then treating it as described above.

HPLC condition: High-performance liquid chromatography (HPLC) was carried out on the Shimadzu LC-6A gradient system, equipped with a Shimadzu RF-535 fluorescence detector (E_x = 300 nm, E_m = 700 nm, sensitivity; high 128) and a Shimadzu C-R4A data processor. A 20 μl of test solution was injected into HPLC. The column used was Inertsil (4.6ϕ × 250 mm, Gasukuro Kogyo Co. Ltd.). The conditions used were as follows: Solvent A, H_2O:AcOH:THF (100:0.2:2) adjusted to pH 5.0 with 2% NH_4OH. Solvent B, H_2O:MeCN:AcOH:THF (10:90:0.2:2)

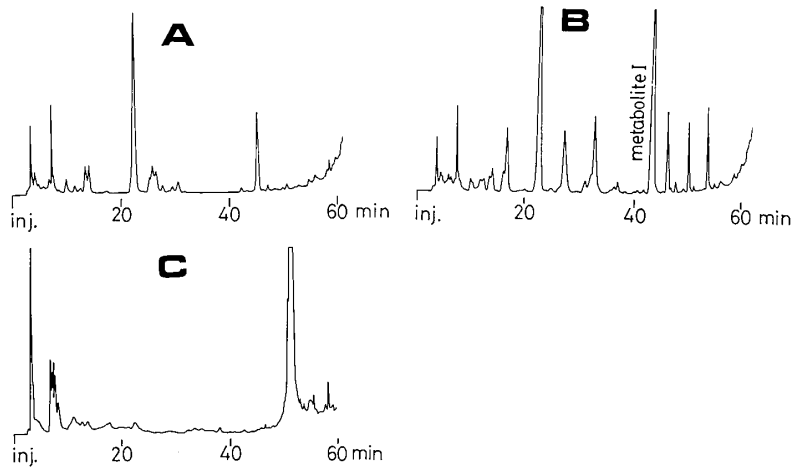

Fig. 1. HPLC-Profile of Rat Urine

A: control urine, B: sample urine (administration: 0.5 ml of 3.0 g/ml of E. Fruit ext.), C: HPLC-profiles of E. Fruit. Condition: column, Inerttsil 4.6 × 250 mm; mobile phase, solvent A = H_2O–AcOH–THF (100:0.2:2, pH 5.0), solvent B = H_2O–MeCN–AcOH–THF (10:90:0.2:2, pH 5.0), A/B = 100/0 → A/B = 10/90, 60 min, curve 5 gradient; flow rate, 1.0 ml/min at 40°C; detective wave length, E_x = 300 nm, E_m = 700 nm; detective sensitivity, high 128.

adjusted to pH 5.0 with 2% NH_4OH. The gradient system was controlled by a Shimadzu system controler SCL-6A and run on curve 5 (A/B = initially 100/0→finally 10/90) within 60 min (1.0 ml/min) at 40°C.

Isolation and identification of metabolite I in urine——Administration: A methanol extract of Evodia Fruit, 0.5 ml of 3.0 g/ml solution (in conversion into crude drug) was orally administered daily for two weeks to 30 rats.

Urine collection: The urine was collected in the manner described above, about 3.0 *l* of rat urine was obtained.

Isolation of metabolites: 3.0 *l* of urine was concentrated to 1.0 *l* under reduced pressure and treated with *n*-butanol. Then the aqueous layer was concentrated to 100 ml under reduced pressure. Then, 900 ml of methanol was added to the concentrated urine and stirred, and the mixture was filtered to remove any insoluble material. The filtrate was then concentrated, treated with methanol and again concentrated. This procedure was repeated three times. Finally, 50 ml of solution freely soluble in methanol was obtained, which was submitted to preparative middle pressure LC. The conditions used were as follows: Solid phase; ODS gel column (22ϕ × 300 mm, Kusano Kagakukikai Co. Ltd.), mobile phase; MeCN:H_2O:AcOH:THF (15:85:0.2:2), flow rate; 10.0 ml/min. The eluate fractions containing metabolite I were collected and concentrated under reduced pressure. Then, aqueous solution was applied to an ODS column and washed with water to remove acetic acid. Then metabolite I was eluted with methanol. The methanol eluate was concentrated in vacuum, and the residue was recrystallized from methanol. About 200 mg of colorless needles was obtained.

Identification of method: Melting point was taken on a Yanaco MP micro melting point apparatus and is uncorrected. UV and IR spectra were run on a Shimadzu UV-3000 spectrometer and on a JASCO FT/IR-7000 spectrometer, respectively. ^1H- and ^{13}C-NMR spectra were recorded on a JEOL GX-270 NMR spectrometer (270 and 67.9 MHz, respectively). Chemical shifts are given on the δ scale with tetramethylsilane as an internal standard (s: singlet, bs: broad singlet, d: doublet, dd: doublet doublet, ddd: doublet doublet doublet, m: multiplet). MS was measured on a Shimadzu 9000-B mass spectrometer.

Results and Discussion

By using HPLC equipped with a fluorescence detector, metabolite I was detected in rat urine after the oral administration of a methanol extract of Evodia Fruit. Metabolite I was presumed to be a main metabolite in the rat urine (Fig. 1). The amount (peak height in HPLC) of metabolite I was directly proportional to the administration doses of the extract (0.5 ml of 1.0 g/ml, 2.0 g/ml and 3.0 g/ml concentration).

Metabolite I (colorless needles, mp 239–240°C uncorrected) showed a molecular ion peak at m/z 259 ($C_{15}H_{17}NO_3$) and fragment ion peaks at m/z 186, 173 and 144. These fragment ion peaks were also detected in the MS of evocarpine[3] (Fig. 2). Its UV spectrum was very similar to that of evocarpine[3] with absorption peaks at 335, 323, 241 and 215 nm (log ε 4.16, 4.13, 4.42 and 4.42). These spectral data suggested that I was a 4-quinolone type alkaloid. The IR spectrum of I showed a conjugated carbonyl band at 1620 cm^{-1} and a carboxylic acid band at 1705 and 3400 cm^{-1}.[7] In the ^1H-NMR spectrum of I, the signals of aromatic pro-

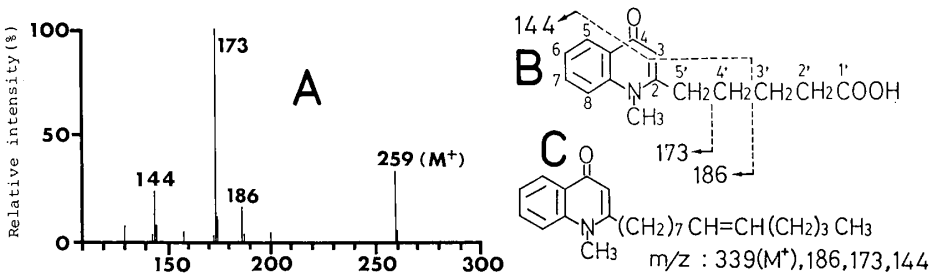

Fig. 2. Structure and Mass Spectrum
A and B: metabolite I, C: evocarpine.

TABLE I. ^{13}C- and ^{1}H-NMR Assignments of Metabolite I

	^{13}C chemical shifts		^{1}H chemical shifts			
C	δ	multiplicity	δ	No. of H-atoms	multiplicity	J(Hz)
2	155.0	s	—			
3	109.8	d	6.04	1H	s	
4	175.7*[a)]	s	—			
4a	125.9	s	—			
5	125.1	d	8.16	1H	dd	7.9, 1.3
6	122.8	d	7.37	1H	ddd	7.9, 6.6, 1.3
7	131.8	d	7.72	1H	ddd	8.6, 6.6, 1.7
8	116.7	d	7.78	1H	d	7.9
8a	141.7	s	—			
N-CH_3	34.2	q	3.74	3H	s	
1′	174.2[a)]	s	—			
2′	33.2	t	2.29	2H	m	
3′	23.9	t	1.63	2H	m	
4′	27.3	t	1.63	2H	m	
5′	33.2	t	2.79	2H	m	

The sample was dissolved in DMSO. All values given relative to TMS.
[a)] Assignments may be reversed.

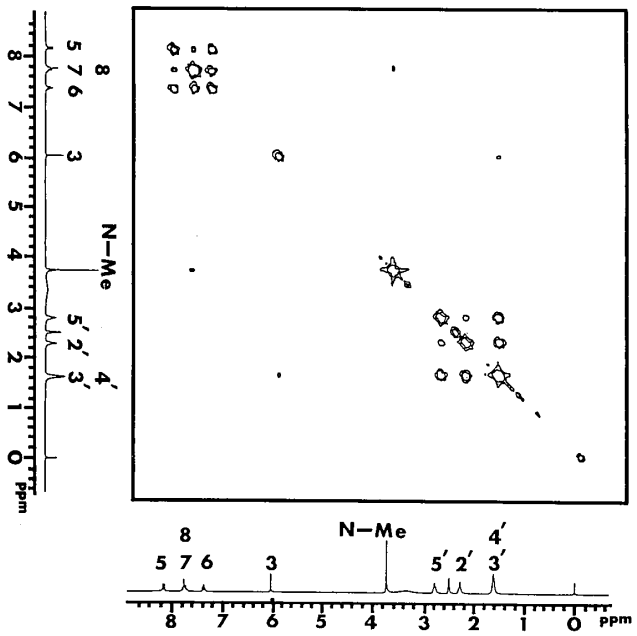

Fig. 3. ^{1}H-^{1}H Shift Correlated Spectroscopy (COSY) of Metabolite I

tons and olefinic proton caused by the 4-quinolone hydrogens were at δ8.16 (dd, $J = 7.9$, $J = 1.3$, C_5-H), δ7.78 (d, $J = 7.9$, C_8-H), δ7.72 (ddd, $J = 8.6$, $J = 6.6$, $J = 1.7$, C_7-H), δ7.37 (ddd, $J = 7.9$, $J = 6.6$, $J = 1.3$, C_6-H) and δ6.04 (s, C_3-H). The *N*-methyl protons resonated at δ3.74 (s, N-CH_3), the methylene protones at $C_{2'}$-H, $C_{3'}$-H, $C_{4'}$-H and $C_{5'}$-H in a high field between δ 2.79 and δ 1.63. The ^{13}C-NMR spectrum of I showed fifteen signals; two carbonyl carbons, six aromatic carbons, two olefinic carbons, one *N*-methyl carbon and four methylene carbons. All the ^{1}H-NMR and ^{13}C-NMR signals were assigned by the 2D-NMR analysis (TABLE I and Fig. 3). The side chain contained four methylenes and a carboxylic acid group at the terminal. From these spectral data, the structure of metabolite I was elucidated to be 5-(1, 4-dihydro-1-methyl-4-oxo-2-quinolin-2-yl)-pentanoic acid (Fig. 2).

Conclusion

A methanol extract of the crude drug Evodia Fruit was orally administered to rat, and 5-(1, 4-dihydro-1-methyl-4-oxo-2-quinolin-2-yl)-pentanoic acid was detected in the rat urine. This metabolite having a 4-quinolone structure seemed to be derived from the evocarpine type compounds[8] contained in the crude drug, Evodia Fruit.

Now, we are investigating the biological relationship between evocarpine and 5-(1, 4-dihydro-1-methyl-4-oxo-2-quinolin-2-yl)-pentanoic acid. We are also trying to identify other peaks in the HPLC-profiles of rat urine.

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