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Urinary metabolites of isorhynchophylline in rats and their neuroprotective activities in the HT22 cell assay

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Abstract

Isorhynchophylline is one of the major alkaloids from the *Uncaria* hook possessing the effects of lowered blood pressure, vasodilatation and protection against ischemia-induced neuronal damage. However, the metabolic pathway of isorhynchophylline has not been fully reported yet. In this paper, the metabolism of isorhynchophylline was investigated in rats. Five metabolites were isolated by using solvent extraction and repeated chromatographic methods, and identified by spectroscopic methods including UV, MS, NMR and CD experiments. Three new compounds were identified as 5-oxoisorhynchophyllilic acid-22-O- β -D-glucuronide (**M1**), 17-O-demethyl-16,17-dihydro isorhynchophylline (**M2**) and 5-oxoisorhynchophyllilic acid (**M4**) together with two known compounds isorhynchophylline (**M0**) and rhynchophylline (**M3**). Possible metabolic pathways of isorhynchophylline are proposed. Furthermore, the activity assay for all the metabolites showed that isorhynchophylline (**M0**) exhibited potent neuroprotective effects against glutamate-induced HT22 cell death. However, little or weak neuroprotective activities were observed for **M1–M4**. Our present study is important to further understand its metabolic fate and disposition in humans.

Keywords

Uncaria hook; Isorhynchophylline; Metabolites from rat urine; Neuroprotection; HT22 cell

1. Introduction

Uncaria hook (UH) is a well-known herbal medicine mentioned in Chinese and Japanese pharmacopeias and has been used to treat hypertension, headache and stroke. It is

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Conflict of interest

No potential conflicts of interest were disclosed.

botanically from the hook-bearing branch of five *Uncaria* plants. Rhynchophylline (RN), isorhynchophylline (IRN, (16E,20 α)-16,17-didehydro-17-methoxy-2-oxo-corynoxan-16-carboxylic acid), corynoxine (CN) and isocorynoxine (ICN) are the four major tetracyclic oxindole alkaloids found in UH [1], and reported to process many beneficial pharmacological effects on cardiovascular and cerebrovascular diseases, such as hypertension [2], vasodilatation [3], anti-platelet aggregation [4], sedation [5,6] and neuroprotection [7,8]. Consistent with the pharmacological activities mentioned above, IRN possesses a blocking activity of calcium release from intracellular stores, a protective effect against the ischemia, glutamate or dopamine-induced damage or death, an inhibitive activity against NMDA receptors expressed, a suppressive effect on 5-HT_{2A} receptor function and others [9]. In recent reports from our laboratories, IRN potently inhibited inflammatory mediators such as NO, TNF- α , and IL-1 β in LPS-activated N9 cells [10]. It is implied that IRN plays an important role in biological activities of UH, especially in neuroprotection. Clinically, in China and Japan, the phytochemicals containing UH, such as yokukansan, tianmagouteng granules and chotosan, have become better selling herbal medicines for therapy of stroke, hypertension, chronic headache and others. According to our previous study [1], the average content of IRN in some raw materials of UH was similar to that of ICN and more than RN and CN. Thus, the information on IRN pharmacokinetics and metabolism is helpful for the clinical applications of the phytochemicals containing UH. Despite wide pharmacological studies of IRN, the metabolism and pharmacokinetics of IRN in humans and other animals remain obscure. To obtain more information about its metabolism to improve its clinical applications, we examined the biotransformation of IRN in rat urine after oral administration of IRN.

The liquid chromatography–tandem mass spectrometry (LC–MSⁿ) technique was applied to characterize the urinary, plasma and/or biliary metabolites of IRN, RN and ICN, the major alkaloids of UH [11–13]. In these studies, the glucuronide-conjugated position of phase II metabolites and the hydroxylation position of phase I metabolites cannot be definitively determined by means of the LC–MSⁿ method alone. Isolation of metabolites and their further structural confirmation on the basis of UV, NMR and mass spectrometry (MS) data are valuable as well. Furthermore, we pay attention to the activity of the metabolites because some phase I metabolites may exert more potent biological activities or side effects than their parent drug [14,15].

The present study describes the isolation and structural elucidation of three new and two known urinary metabolites in rats orally given IRN, and the neuroprotection of IRN and its metabolites (**M1–M4**) against 3.0 mM glutamate-induced oxidative cell death in HT22 mouse hippocampal cells. Moreover, the possible metabolic pathway of IRN was proposed on the basis of the metabolite profile.

2. Materials and methods

2.1. General

Macroporous resin AB-8 was obtained from the Nankai University Chemical Factory (Tianjin, China); Sephadex LH-20 was from GE Healthcare (Uppsala, Sweden); ODS was obtained from YMC Co., Ltd. (Kyoto, Japan); and silica gel GF₂₅₄ for thin-layer

chromatography was from Qindao Ocean Chemical Co. (Qingdao, China). Other chemical reagents were of analytical or HPLC grade. Double-distilled water from Jinzhou Scientific Instrument Co., Ltd. (Tianjin, China) was used in this study.

2.2. Plant material

Leaves of *Uncaria rhynchophylla* were collected in October 2005 in Fujian Province, China. A voucher specimen is maintained in the Department of Traditional Chinese Medicines, Shenyang Pharmaceutical University. Species identification was confirmed by Professor Zerong Jiang, Shenyang Pharmaceutical University. IRN and RN were isolated from the leaves of *U. rhynchophylla* (Miq.) Jacks, following the previously reported methods by Yuan et al. [8] and Ma et al. [16]. The identity of these compounds was confirmed by melting point, UV, IR, ^1H and ^{13}C NMR and MS. The purity of IRN and RN evaluated with high-performance liquid chromatography was more than 98.6%.

2.3. Animals

Male Wistar rats (200 ± 20 g b.wt.) were purchased from the Animal Center of Shenyang Pharmaceutical University (Shenyang, China). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Shenyang Pharmaceutical University. They were kept in a breeding room to be acclimated for 4 days before use. Normal foods were available before experiments, and normal water was available at all times.

2.4. Urine collection

Male Wistar rats were fasted 12 h before experiments. IRN (0.8 g) was given orally to 15 rats at a dose of $40 \text{ mg}\cdot\text{kg}^{-1}$ (dissolved into 0.1 M HCl , and immediately diluted to $10 \text{ mg}\cdot\text{mL}^{-1}$ with water, $\text{pH} = 6.0\text{--}6.5$) and administered reiteratively in an interval of 6 days (1 day for administration and 5 days for recovery) for the collection of urine samples. The urine samples were collected from 0 to 24 h. During the collection, water and sugar were available freely. Urine samples were subsequently placed in the refrigerator at -20°C .

2.5. Extraction and isolation

The cumulative urine samples (approximately 1.8 L in total) were thawed at room temperature and successively passed through a macroporous absorption resin AB-8 column eluted with a gradient of $\text{EtOH-H}_2\text{O}$ (H_2O , 50% $\text{EtOH-H}_2\text{O}$, 70% $\text{EtOH-H}_2\text{O}$, and 95% $\text{EtOH-H}_2\text{O}$ elutions) to yield four major fractions (Fr.1–4).

The 50% $\text{EtOH-H}_2\text{O}$ fraction (Fr.2) was passed through a Sephadex LH-20 column (35×800 mm) and eluted with $\text{MeOH-H}_2\text{O}$ (50:50), and the elutes were grouped on the basis of TLC analysis into two fractions (Fr.2-1 and Fr.2-2). The fraction (Fr.2-2) was further subjected to a Sephadex LH-20 column (15×500 mm) eluting with $\text{MeOH-H}_2\text{O}$ (50:50) to yield one major fraction. Finally, pre-HPLC was carried out using $\text{MeOH-H}_2\text{O}$ (15:85) plus 0.03% Et_2NH as an eluent at a flow rate of $1 \text{ mL}\cdot\text{min}^{-1}$ to yield **M1** (10.0 mg).

The 70% $\text{EtOH-H}_2\text{O}$ (Fr.3) fraction was passed through a Sephadex LH-20 column (35×800 mm) chromatograph and a linear gradient of $\text{MeOH-H}_2\text{O}$ (70:30–100:0) to yield three

major fractions (Fr.3-1, Fr.3-2, and Fr.3-3). Fraction Fr.3-2 was further separated through an ODS open column eluting with a gradient of MeOH–H₂O (20:80–100:0) to yield three major fractions (Fr.3-2-1–Fr.3-2-3). The fraction (Fr.3-2-1) eluted with MeOH–H₂O (40:60) was further subjected to a pre-HPLC eluting with MeOH–H₂O (35:65) plus 0.03% Et₂NH to yield **M3** (2.1 mg) and **M0** (1.8 mg). Fraction Fr.3-2-2 eluted with MeOH–H₂O (40:60) was further subjected to a pre-HPLC eluting with MeOH–H₂O (40:60) plus 0.03% Et₂NH to yield **M2** (13.0 mg). Fraction Fr.3-2-3 eluted with MeOH–H₂O (70:30) was further subjected to a pre-HPLC eluting with MeOH–H₂O (45:55) plus 0.03% Et₂NH to yield **M4** (8.0 mg).

2.5.1. 5-Oxoisorhynchophylllic acid-22-O-β-D-glucuronide (M1)—White amorphous powder (MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 210 (5.16), 244 (4.85), and 281 (3.92); and ¹H NMR and ¹³C NMR (DMSO) data are shown in Table 1; CD (0.72 mM, MeOH, 25 °C); ESI-QTOFMS (positive) m/z 561.2069 (calculated as 561.2071).

2.5.2. 7-O-demethyl-16,17-dihydro isorhynchophylline (M2)—White amorphous powder (MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 211 (5.14), 252 (4.79), and 291 (3.88); and ¹H NMR and ¹³C NMR (DMSO) data are shown in Table 1; CD (0.58 mM, MeOH, 25 °C); ESI-QTOFMS (positive) m/z 387.1924 (calculated as 387.1920).

2.5.3. 5-Oxoisorhynchophylllic acid (M4)—White amorphous powder (MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 210 (5.16), 243 (4.81), and 281 (3.90); and ¹H NMR and ¹³C NMR (DMSO) data are shown in Table 1; CD (0.63 mM, MeOH, 25 °C); ESI-QTOFMS (positive) m/z 385.1735 (calculated as 385.1738).

2.6. Cell culture

HT-22 cells were cultured in DMEM (HyClone, Logan, UT, USA) supplemented with 10% FBS (Atlanta Biological, Lawrenceville, GA, USA) at 37 °C in an atmosphere containing 5% CO₂ and 95% air [17]. HT-22 cells were obtained from David Schubert (Salk Institute, San Diego, CA, USA).

2.7. Cell viability assay

Cell viability was determined in HT-22 cells by the calcein AM cell viability assay [17]. HT-22 cells were seeded for 24 h before initiation of the experiment at a density of 5000 cells per well in 96-well plates. Insult cells with 3.0 mM glutamate induce 50–75% cell death and co-treat with IRN and **M1–M4** at the concentrations of 100 nM, 1 μ M, 10 μ M, 50 μ M and 100 μ M, and 2-(1-adamantyl)-4-methylestrone (ZYC26) at the concentrations of 100 nm, 1 μ M, 5 μ M and 10 μ M for 24 h. After exposure to various treatment paradigms, cells were rinsed with PBS, and cell viability was measured using the membrane-permeant calcein-AM dye (Molecular Probes, Eugene, OR, USA). Calcein-AM is a fluorogenic esterase substrate that is hydrolyzed to a fluorescent product in cells having esterase activity and intact membranes. Cells were incubated in a solution of 1 μ M calcein-AM in PBS at 37 °C in the dark. Twenty minutes later, fluorescence was determined using a Bio-Tek (Winooski, VT, USA) FL600 microplate reader with an excitation/emission filter set of

485/530 nm. The results, obtained in relative fluorescent units, are expressed as the percentage of untreated control values.

2.8. Spectroscopic methods

NMR spectra were measured on a Bruker ARX-300, 400 and 500 MHz spectrometer (Bruker, Switzerland), and chemical shifts are given in ppm downfield relative to tetramethylsilane (TMS) as the internal standard. All compounds were dissolved in dimethyl sulfoxide-d₆ or CDCl₃. UV spectra were obtained using a Shimadzu UV-2201 spectrophotometer (Shimadzu, USA). Circular dichroism (CD) spectra were recorded on a Bio-Logic modular optical system 450 (MOS-450; Bio-Logic; France).

2.9. Chemical analysis

2.9.1. Q-TOF/MSE parameters—Analyses were performed using a Micromass-Q-TOF Premier mass spectrometer (Waters Corp., Milford, MA, USA) coupled with an electrospray ionization (ESI) source operated in positive ion mode. There are sensitivity and resolution mode available for acquiring mass data. For ions originating from a given source and accelerated by a fixed potential, the mass resolving power of a TOF-MS will increase as the flight path is lengthened. However, a longer flight path will sure reduce the total signal as fewer ions strike the detector. Therefore, the sensitivity mode is more sensitive, while the resolution mode offers a higher mass resolution. In the present study, the sensitivity mode was used for the detection of some trace amount of metabolites in biological samples. The MS tune parameters were as follows: the cone and desolvation gas flows were 50 L/h and 800 L/h, respectively; the temperatures of the source and desolvation were set at 130 °C and 450 °C, respectively; the capillary and the cone voltages were set at 3.0 kV and 40 eV, respectively; the micro-channel plates (MCPs) were operated at 1750 V and the Q-TOF mass spectrometer was operated in MSE mode with a low collision energy set at 6 eV in the first function and a collision energy ramp from 25 to 40 eV in the second function. Centroid mode data were collected over the range of *m/z* 100–1000 in both functions, and the scan time was 0.2 s with an inter-scan delay of 0.02 s. For the dynamic range enhancement (DRE) lock mass, a 2 ng/mL solution of leucine-enkephalin generating an [M + H]⁺ ion (*m/z* 556.2771) was infused through the Lock Spray probe at 10 µL/min.

2.9.2. UHPLC conditions—Chromatographic separation was performed on a Waters ACQUITY UPLC™ system (Waters Corp., Milford, MA, USA) using a Waters ACQUITY HSS C18 column (100 mm × 2.1 mm i.d., 1.8 µm). The oven temperature was maintained at 35 °C. The temperature of auto-sampler was fixed at 10 °C. Water containing 0.1% formic acid served as solvent system A and acetonitrile served as solvent system B. The flow rate was 0.5 mL/min, and a 2 µL injection volume with partial loop using needle overfill (PLNO) mode was used. The mobile phase with a 12-min gradient elution of 5–15% B from 0 to 1.5 min, 15–20% B from 1.5 to 4.5 min, 20–25% B from 4.5 to 5 min, 25–30% B from 5 to 6.5 min, 30–35% B from 6.5 to 9 min, 35–40% B from 9 to 10 min, 40–5% B from 10 to 11 min, and 5% B from 11 to 12 min was used.

3. Results

3.1. Isolation and structural elucidation of IRN metabolites

Representative UHPLC profiles showing the rat urinary metabolites are given in Fig. 1. IRN and its metabolites were selectively detected at 245 nm due to their characteristic oxindole nucleus group. Five major metabolites were clearly observed in rat urine comparing with blank urine. By means of repeated chromatographic methods on the columns of macroporous resin AB-8, Sephadex LH-20, reverse-phase ODS, and pre-HPLC, they were isolated from the rat urine sample, including three new compounds, **M1**, **M2** and **M4**, together with two known alkaloids **M0** and **M3**.

M1 was isolated as a white amorphous powder. The molecular formula was determined to be $C_{27}H_{32}O_{11}$ from the $[M + H]^+$ quasi molecular ion peak at m/z 561.2069 (calculated 561.2071) in the ESI-QTOF-MS. The $[M + H]^+$ ion at m/z 561 and an important fragment ion at m/z 385 originating from the eliminating 176 mass units (glucuronic acid) from $[M + H]^+$ ion indicated that **M1** should be a glucuronide conjugate. 1H -NMR (300 MHz, $DMSO-d_6$) spectra gave a group of o-substituted benzene ring proton signals (δ_H 7.21, 1H, t, $J = 7.5$ Hz, 7.14, 1H, d, $J = 7.2$ Hz, 7.01, 1H, t, $J = 7.5$ Hz and 6.87, 1H, d, $J = 7.5$ Hz), which were in connection with four aromatic carbon signals (δ_C 128.5, 124.1, 122.1 and 110.0) in HSQC spectra. ^{13}C -NMR (75 MHz, $DMSO-d_6$) spectra gave a lactam carbonyl (δ_C 178.7), two aromatic quaternary carbons (δ_C 130.4 and δ_C 141.7) and quaternary carbon (δ_C 51.1) signals. These data indicate that **M1** possesses an oxindole structure. Moreover, the resonances for the carbons and protons in the 1H and ^{13}C NMR as well as HSQC spectra included four methylene (δ_H 2.38, 2.71, δ_C 41.3; δ_H 0.93, 1.73, δ_C 31.2; δ_H 0.93, 1.35, δ_C 23.4 and δ_H 2.42, 4.06, δ_C 44.4), two methines (δ_H 2.54, δ_C 36.5; δ_H 1.89, δ_C 36.3), a methane connected to N atom or O atom (δ_H 3.66, δ_C 62.1), a methoxy (δ_H 3.79, 3H, s, δ_C 61.7), an sp^2 methine (δ_H 7.46, 1H, s, δ_C 168.1) that should be directly attached to oxygen, an sp^2 quaternary carbon (δ_C 109.7), and two carbonyls (δ_C 170.3 and 165.3). HMBC spectra gave some correlations that were determined between H-6 and C-2, C-3, C-5, C-7, and C-8, between H-3 and C-2, C-7 and C-8, between H-21 and C-3, C-15 and C-20 and between H-15 and C-20, respectively. Its NMR data (Table 1) suggested that its tetracyclic framework was similar to that of IRN except that C-5 of **M1** was a carbonyl (δ_C 170.3). Moreover, the remaining molecular formula $C_6H_9O_3$ consisted structurally of two chain substituents, being attached to the cyclic framework separately at C-15 and C-20. The constitution of one of the substituents, $C_4H_4O_3$, and its location at C-15 were deduced from the HMBC spectrum. HMBC correlations of the substituents were determined between the methoxy protons and C-17 and between H-17 and C-15, C-16, C-22 and methoxy carbon, respectively. Subsequently, the remaining substituent, an ethyl group ($-CH_2CH_3$), could be attached only to C-20, as evidenced by the appreciable correlation between H-18 and C-20 in the HMBC experiment. The signals of an anomeric proton and carbon (δ_H 5.19, 1H, d, $J = 7.5$ Hz, δ_C 93.8) and a carboxylic group at C-6' at δ_C 172.0 indicated the presence of a β -configuration of the glucuronic acid moiety [18]. The correlation between an anomeric proton and C-22 in the HMBC experiment of **M1** indicated that a β -D-glucuronic acid moiety could be attached to the C-22 position, as evidenced by the upfield shift of C-22 (-9.2 ppm) relative to the corresponding signals of **M4**. The full assignments of proton and

carbon signals are summarized in Table 1. The absolute configuration of **M1** was determined from the CD spectrum and 2D NOESY correlation (Fig. 3-A). As a tetracyclic oxindole alkaloid, its 7S configuration and α -orientation of H-3 were deduced on the basis of the presence of a negative CE at 285 nm, a positive CE at 220 nm, and a negative CE at 265 nm in the CD data, respectively [19,20]. The α -orientation of H-15 was indicated by the strong NOESY correlation of H-3/H-15. The β -orientation of H-20 was indicated by the strong NOESY correlations of H-20/H-9 and H-9/ β -H-14. Thus, Metabolite **M1** possesses 7S, 3S, 15S, and 20R absolute configurations, which are consistent with the allo A configuration for IRN [20]. **M1** was determined to be 5-oxoisorhynchophylllic acid-22-O- β -D-glucuronide.

M2 was isolated as a white amorphous powder. The molecular formula was determined to be $C_{21}H_{26}N_2O_5$ from the $[M + H]^+$ quasi molecular ion peak at m/z 387.1924 (calculated as 387.1920) in the ESI-QTOF-MS. Its NMR data (Table 1) suggested that its tetracyclic framework was the same to that of **M4**. The constitution and location of both substituents $C_4H_7O_3$ and C_2H_5 were also deduced from the HMBC spectrum. For **M2**, the 7S configuration and α -orientation of H-3 were deduced on the basis of the presence of a negative CE at 285 nm, a positive CE at 220 nm, and a negative CE at 265 nm in the CD data, respectively [19,20]. The α -orientations of H-15 and H-16 were indicated by the strong NOESY correlations of H-3/H-15 and α -H-14/H-16. The β -orientation of H-20 was indicated by the strong NOESY correlations of H-20/ β -H-14 (Fig. 3-B). Thus, **M2** possesses 7S, 3S, 15S, 16S, and 20R absolute configurations, which are consistent with the allo A configuration for IRN [20]. **M2** was determined to be 17-O-demethyl-16,17-dihydro isorhynchophylline.

M4 was isolated as a white amorphous powder. The molecular formula was determined to be $C_{21}H_{24}N_2O_5$ from the $[M + H]^+$ quasi molecular ion peak at m/z 385.1735 (calculated as 385.1738) in the ESI-QTOF-MS. This implies that it has 11° of unsaturation. The molecular weight of **M4** was the same as that of IRN (**M0**, $C_{22}H_{28}N_2O_4$), but its molecular composition was different from that of IRN. Its NMR data (Table 1) suggested that its tetracyclic framework was the same to that of **M1**. The constitution and location of both substituents $C_4H_5O_3$ and C_2H_5 were also deduced from the HMBC spectrum. For **M4**, the 7S configuration and α -orientation of H-3 were deduced on the basis of the presence of a negative CE at 285 nm, a positive CE at 220 nm, and a negative CE at 265 nm in the CD data, respectively [19,20]. The α -orientation of H-15 was indicated by the strong NOESY correlation of H-3/H-15. The β -orientation of H-20 was indicated by the strong NOESY correlations of H-20/H-9 and H-9/ β -H-14 (Fig. 3-A). Thus, Metabolite **M4** possesses 7S, 3S, 15S, and 20R absolute configurations, which are consistent with the allo A configuration for IRN [20]. **M4** was determined to be 5-oxoisorhynchophylllic acid.

M0 and **M3** were identified as isorhynchophylline and rhynchophylline [21,22], respectively, by comparing their UV, MS and tR data with standard compounds. **M3** was first isolated as the metabolites of IRN from rat urine.

3.2. Neuroprotection of IRN and metabolites in the HT22 cell assay

The neuroprotective effect of some metabolites of IRN, together with **M1–M4**, against 3.0 mM glutamate-induced HT22 cell death was investigated. Cell viability was determined in HT-22 cells by the calcein AM cell viability assay [17]. As shown in Fig. 4-A and -B, IRN exhibited potent neuroprotective effects against glutamate-induced cell death. However, **M1–M4** were little or weak neuroprotective activities at the maximum concentration (100 μ M).

4. Discussion

The metabolic pathways of indole alkaloids in vivo are related to many reactions such as hydrolysis, O-demethylation [23], glucuronidation [12,13], sulfation [24,25], hydroxylation [26–28], reduction [29] and others [30]. In the present study, three phase I metabolites (**M2–M4**), one phase II metabolite (**M1**) and IRN (**M0**) itself were isolated from the urine of rats given IRN orally, and structurally confirmed on the basis of UV, NMR, MS and CD data. Also the neuroprotection of IRN and its metabolites, **M1–M4**, against 3.0 mM glutamate-induced HT22 cell death was investigated. According to the metabolite profile, the possible metabolic pathways of IRN in rats are proposed as shown in Fig. 2.

M1, **M2** and **M4** are three major metabolites. **M4** is believed to be formed through the oxidation at C-5 and hydrolysis of 22-carboxylic methyl ester catalyzed by hepatic cytochrome P450 enzyme [23,31–35]. Moreover, **M4** is also a reactive metabolite that can be successively converted to a glucuronide-conjugate (**M1**) via further glucuronidation at 22-carboxylic group catalyzed by UDP-glucuronosyltransferases [23]. Most importantly, we found that **M2** is another key metabolite derived from the oxidation at C-5 and reduction of the 16-double bond together with an O-demethylation at C-17 catalyzed by cytochrome P450 [25,29,36]. Wang et al. [12,13] reported that the hydroxylation at C-10 or C-11 of the aryl group was the major metabolic pathway after oral administration of IRN or RN dissolved in DMSO at a single dose of 37.5 mg/kg b.wt. to Wistar rats. In the present study, 5-oxo-IRN derivatives were found as major metabolites in rat urine after oral administration of IRN dissolved in 0.1 M HCl solvent at a single dose of 40.0 mg/kg b.wt. to male Wistar rats. The oxidation at C-5 is the major metabolic pathway for IRN, which was found in the metabolism of nicotine [32,35] and (–)-N-{2-[(R)-3-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline-2-carbonyl)piperidino]ethyl}-4-fluorobenzamide [34]. We conducted a comparative experiment in order to explain the difference in major metabolites. The comparative assay showed that peak areas of 10 or 11-hydroxy-IRN in urine samples of rats orally given IRN dissolved in 0.1 M HCl solvent were lower than those in DMSO, while peak areas of 5-oxo-IRN in urine samples of rats orally given IRN dissolved in 0.1 M HCl solvent were higher than those in DMSO, respectively. It is worth noting that the oral administration of the alkaloid hydrochloride salt to rats is closer to clinical application. Firstly, the alkaloid hydrochloride salt could be formed in the acidic environment of the gastrointestinal tract after oral administration of the alkaloid. Secondly, the phytochemicals containing the chloride salts of rhynchophyllinoid alkaloids have been used for several decades in clinical situations to treat hypertension and headache in China, such as total alkaloids of *U. rhynchophylla* (Miq.) Jacks. tablets. Thus, the oxidation at C-5 should be the

major metabolic pathway for tetracyclic oxindole alkaloids, which is reported for the first time.

In addition, a pair of epimers (**M0** and **M3**) was obtained. **M3** was the epimer of **M0** that is believed to be formed through the isomerization at C-7 oral administration. RN is reported to undergo isomerization at the spiro center to form IRN in rat plasma after intravenous administration of RN [13]. However, no isomerization was observed in vivo after oral administration of RN. In fact, the isomerization at the spiro center of rhynchophylline-type oxindole alkaloids in vitro was recognized and a retro-Mannich ring opening, rotation and Mannich ring closure were proposed as the mechanism [37]. This implied that the isomerization at the spiro center in vivo may be undergoing without enzymatic conversion.

Glutamate is a mammalian central nervous system neurotransmitter in normal neuronal cells. However, high concentrations of glutamate produce oxidative stress, resulting in neurodegeneration such as in stroke, trauma, Alzheimer's disease, and Parkinson's disease [38–40]. HT22 cells, an immortalized mouse hippocampal cell line, have been widely used in recent years as an in vitro model for studying the mechanism of glutamate-induced neurotoxicity [17,41]. The neuroprotective effects of all the metabolites, together with IRN, against 3.0 mM glutamate-induced HT22 cell death were investigated. The cytotoxic activity of IRN and its metabolites on HT22 cells in the absence of glutamate was assessed by the calcein AM cell viability assay. Co-treatment of unstimulated HT22 cells with all samples did not affect the cell viability at the tested concentrations of 100 nM, 1 μ M, 10 μ M, 50 μ M and 100 μ M (data not shown). As shown in Fig. 4-A and -B, the activity assay showed that IRN exhibited a potent neuroprotective effect against glutamate-induced cell death. In a parallel experiment, ZYC26, an estrogen-like compound, significantly possesses a neuroprotective against glutamate-induced cell death [42]. However, little or weak neuroprotective activities were observed for **M1–M4**. The C-7 configuration of IRN and RN seemed to affect their neuroprotective activities, which is consistent with the report that antagonistic activity of alkaloids from UH on 5-HT_{2A} receptors in the brain is closely related to the 7S configuration of the oxindole moiety. It was apparent that IRN is initially administered to the rat in an active form, and then becomes converted to its inactive forms excreted in urine through some in vivo biotransformations such as the oxidation at C-5, reduction of the 16-double bond, O-demethylation at C-17, hydrolysis at 22-carboxylic methyl ester and/or isomerization at C-7. It is usual for the alkaloid to be eventually eliminated in urine in its inactive (or less than fully active) form in vivo.

In summary, three new metabolites together with two known ones were isolated from the urine of rats given IRN orally and structurally confirmed by UV, NMR, MS, and CD techniques. It is found for the first time that the major metabolic pathways for the tetracyclic oxindole alkaloids should be the oxidation at C-5 and hydrolysis at 22-carboxylic methyl ester. The formed metabolites were excreted either free or conjugated.

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Abbreviations

UH	<i>Uncaria hook</i>
RN	rhynchophylline
IRN	isorhynchophylline
CN	corynoxine
ICN	isocorynoxine
ZYC26	2-(1-adamantyl)-4-methylestrone

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Fig. 1. UHPLC–UV chromatograms of blank urine (A), a rat urine sample during 0 to 24 h after oral administration of 40 mg/kg of IRN (B), and metabolite standards (C).

Fig. 2.
Proposed metabolic pathways of IRN.

(A) M1 R=COOGlu
M4 R=COOH

(B) M2

Fig. 3.
Key NOESY correlations of **M1** and **M4** (A), and key NOESY correlation of **M2** (B).

Fig. 4.

Effects of IRN and some metabolites against 3.0 mM glutamate-induced HT22 cell death. 1) HT22 cell treated simultaneously with 3.0 mM glutamate. 2) Cell viability was determined by calcein AM assay after 24 h of exposure to the various samples. 3) All data were normalized to percentage survival of control. 4) Data are represented as mean $\pm$ SEM for $n = 8$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus glutamate-treated group alone.

Table 1

¹H and ¹³C NMR data of M1, M2 and M4.

Position	M1 ^a		M2 ^b		M4 ^c	
	δ_C^d , mult.	δ_H (J in Hz)	δ_C^d , mult.	δ_H (J in Hz)	δ_C^d , mult.	δ_H (J in Hz)
2	178.7, qC		179.1, qC		178.8, qC	
3	62.1, CH	3.66, m	62.4, CH	3.76, m	62.6, CH	3.64, m
5	170.3, qC		171.3, qC		170.1, qC	
6	41.3, CH ₂	2.38, d β (16.5)	41.6, CH ₂	2.53, d β (16.8)	40.8, CH ₂	22.43, d β (16.8)
		2.71, d α (16.5)		3.03, d α (16.8)		2.71, d α (16.8)
7	51.1, qC		52.0, qC		50.8, qC	
8	130.4, qC		130.4, qC		130.3, qC	
9	124.1, CH	7.14, d (7.2)	124.2, CH	7.16, d (7.6)	124.1, CH	7.13, d (6.8)
10	122.1, CH	7.01, t (7.5)	122.8, CH	7.05, t (7.2, 7.6)	121.6, CH	7.00, t (7.2)
11	128.5, CH	7.21, t (7.5)	129.0, CH	7.27, m	128.4, CH	7.22, t (7.2, 8)
12	110.0, CH	6.87, d (7.5)	110.6, CH	6.92, d (7.6)	109.9, CH	6.88, d (8)
13	141.7, qC		140.2, qC		141.7, qC	
14	31.2, CH ₂	0.93, m α	29.1, CH ₂	1.01, m β	30.7, CH ₂	0.81, m α
		1.73, m β		1.26, m α		1.75, m β
15	36.5, CH	2.54, m	39.3, CH	1.61, m	37.1, CH	2.45, m
16	109.7, qC		47.4, qC	2.90, m	109.9, qC	
17	161.8, CH	7.46, s	61.7, CH	3.50, m	159.9, CH	7.28, s
				3.80, m		
18	10.5, CH ₃	0.81, t (6.9)	10.4, CH ₂	0.95, t (7.6)	10.7, CH ₃	0.81, t (7.6)
	23.4, CH ₂	0.93, m	22.9, CH	1.20, m	23.3, CH ₂	0.95, m
19		1.35, m		1.76, m		1.35, m
20	36.3, CH	1.89, m	38.2, CH	1.41, m	36.5, CH	1.88, m
21	44.4, CH ₂	2.42, m α	44.7, CH ₂	2.44, m α (12.8)	44.3, CH ₂	2.40, m α
		4.06, d β (8.7)		4.28, dd β (4.4, 13.2)		4.06, dd β (4.4, 12.8)
22	165.3, qC		173.5, qC		174.5, qC	
17-OCH ₃	61.7, CH ₃	3.79, s			61.3, CH ₃	3.73, s

Position	M1 ^a		M2 ^b		M4 ^c	
	δ_{C}^d , mult.	δ_{H} (J in Hz)	δ_{C}^d , mult.	δ_{H} (J in Hz)	δ_{C}^d , mult.	δ_{H} (J in Hz)
22-OCH ₃			51.5, CH ₃	3.56, s		
NH		10.61 br, s		8.70 br, s		10.6 br, s
1'	93.8, CH	5.19, m				
2'	72.4, CH	3.14, m				
3'	76.5, CH	3.20, t (8.7, 8.4)				
4'	71.9, CH	3.12, m				
5'	74.3, CH	3.28, d (9.6)				
6'	172.0					

^a 300 MHz for ¹H and 75 MHz for ¹³C in DMSO.

^b 400 MHz for ¹H and 100 MHz for ¹³C in CDCl₃.

^c 400 MHz for ¹H and 100 MHz for ¹³C in DMSO.

^d Multiplicity and assignment from HSQC experiment.