Published in final edited form as:

Planta Med. 2015 January ; 81(1): 46–55. doi:10.1055/s-0034-1383357.

Isolation and Identification of Twelve Metabolites of Isocorynoxine in Rat Urine and their Neuroprotective Activities in HT22 Cell Assay

Wen Qi¹, Fangfang Chen¹, Jiahong Sun², James W. Simpkins², and Dan Yuan¹

¹Department of Traditional Chinese Medicines, Shenyang Pharmaceutical University, Shenyang, People's Republic of China

²Center for Basic and Translational Stroke Research, Department of Physiology and Pharmacology, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, United States

Abstract

Isocorynoxine, one of the major alkaloids from *Uncaria Hook*, shows the effects of lowering blood pressure, vasodilatation, and protection against ischemia-induced neuronal damage. In this paper, the metabolism of isocorynoxine was investigated in rats. Twelve metabolites and the parent drug were isolated by using solvent extraction and repeated chromatographic methods, and determined by spectroscopic methods including UV, MS, NMR, and CD experiments. Seven new compounds were identified as 11-hydroxyisocorynoxine, 5-oxoisocorynoxineic acid-22-*O*- β -D-glucuronide, 10-hydroxyisocorynoxine, 17-*O*-demethyl-16,17-dihydro-5-oxoisocorynoxine, 5-oxoisocorynoxineic acid, 21-hydroxy-5-oxoisocorynoxine, and oxireno[18,19]-5-oxoisocorynoxine, together with six known compounds identified as isocorynoxine, 18,19-dehydrocorynoxineic acid, 18,19-dehydrocorynoxineic acid B, corynoxine, isocorynoxine-*N*-oxide, and corynoxine-*N*-oxide. Possible metabolic pathways of isocorynoxine are proposed. Furthermore, the activity assay for the parent drug and some of its metabolites showed that isocorynoxine exhibited a significant neuroprotective effect against glutamate-induced HT22 cell death at the maximum concentration. However, little or weak neuroprotective activities were observed for M-3, M-6, M-7, and M-10. Our present study is important to further understand their metabolic fate and disposition in humans.

© Georg Thieme Verlag KG Stuttgart New York

Correspondence: Prof. Dr. Dan Yuan, Shenyang Pharmaceutical University, Department of Traditional Chinese Medicine, 103 Wenhua Road, Shenyang, 110016, China, Phone: + 862423986502, Fax: + 862423986502, yuandan_kampo@163.com. Co-correspondence: Prof. Dr. James W. Simpkins, Robert C. Byrd Health Sciences Center, West Virginia University, Center for Basic and Translational Stroke Research, Department of Physiology and Pharmacology, Morgantown, WV 26506, United States, Phone: + 13042937430, Fax: + 13042933850, jwsimpkins@hsc.wvu.edu.

Conflict of Interest

The authors have no conflict of interest to report.

Supporting information available online at <http://www.thieme-connect.de/products>

Supporting information

ESI-QTOF-MS data of M-3, M-4, M-6, M-7, and M-8 as well as ¹H NMR data of M-0 and its metabolites M-1, M-5, M-6, M-8, and M-9 can be found as Supporting Information.

Keywords

Uncaria Hook; Rubiaceae; isocorynoxine; metabolites from rat urine; neuroprotective activities; HT22 cell

Introduction

UH is a well-known herbal medicine mentioned in Chinese and Japanese Pharmacopoeias, which has been used to treat hypertension, headache, and stroke. It is derived botanically from the branches with hooks of five plants of the genus *Uncaria* (Rubiaceae). RN, IRN, CN, and ICN [(16*E*,20*a*)-16,17,18,19-tetrahydro-17-methoxy-2-oxocorynoxan-16-carboxylic acid methyl ester] are the four major tetracyclic oxindole alkaloids found in UH [1] and reported to process many beneficial pharmacological effects on cardiovascular and central nervous system diseases, such as hypertension [2], vasodilatation [3], anti-platelet aggregation [4], sedation [5,6], and neuroprotection [7,8]. Consistently with the pharmacological activities mentioned above, it has been reported that ICN has a protective effect against glutamate-induced neuronal death in cultured cerebellar granule cells by inhibition of Ca²⁺ damage in the rat hippocampus [7], a suppressive effect on 5-HT_{2A} receptor function [9], and a suppressive activity against lipopolysaccharide-induced NO release in primary cultured rat cortical microglia [8]. It is implied that ICN plays an important role in the biological activities of UH, especially in neuroprotection. Clinically, in China and Japan, the phytochemicals containing UH, such as Tianmagouteng granules, Yokukansan, and Chotosan, have become best-selling herbal medicines for therapy of stroke, hypertension, chronic headache, and others. According to our previous study [1], the average content of ICN in some raw materials of UH was similar to that of IRN and more than RN and CN. Thus, the information on ICN metabolism is helpful for the clinical applications of the phytochemicals containing UH. Despite wide pharmacological studies of ICN, the metabolism and pharmacokinetics of ICN in human and other animals remain obscure. Huang et al. [10] investigated biotransformation in rats orally given ICN, and found four phase II metabolites in rat bile although they were not identified. Also the incubation of the substrates with rat liver microsomes shows that CYP450 plays a key role in their metabolic catalysis. ICN was found by Cai et al. [11] in the plasma of rats which received ICN orally. Wang et al. [12] found that CN was detected in bile, together with its four metabolites, namely, 10 or 11-hydroxy-CN and their 10 or 11-*O*-glucuronides, after oral administration of CN to rats. In other studies by Wang et al. [13,14], an LC-MS examination detected RN or IRN in plasma, bile, urine, and feces, 10 or 11-*O*- β -D-glucuronides of RN or IRN in bile, and 10 or 11-hydroxy-RN or IRN in urine and feces, respectively, after oral administration of RN or IRN. Yu et al. [15] proposed that the metabolite of IRN in cat plasma was formed from 16-double-bond reduction after intravenous administration of IRN. The results mentioned above indicate that the metabolism of ICN may be much more complicated than that of RN and IRN due to its vinyl group at C-20.

The liquid chromatography-tandem mass spectrometry (LC-MSⁿ) technique was applied to characterize the urinary, plasma, and/ or biliary metabolites of ICN, IRN, and RN, the major alkaloids of UH [10,13,14]. In these studies, the glucuronide-conjugated position of phase II

metabolites and the hydroxylation position of phase I metabolites could not 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 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 [16,17].

The present study describes the isolation and structural elucidation of seven new and six known compounds in urine of rats orally given ICN, and the neuroprotection of ICN and its major metabolites against glutamate-induced HT22 cell death. Moreover, a possible metabolic pathway of ICN was proposed on the basis of the metabolite profile.

Results and Discussion

Representative UHPLC profiles showing the rat urinary metabolites are given in Fig. 1. ICN and its metabolites were selectively detected at 245 nm due to their characteristic oxindole nucleus group. Twelve major metabolites together with the parent drug were clearly observed in rat urine compared to the blank control. By means of repeated chromatographic methods on columns of macroporous resin AB-8, Sephadex LH-20, reverse-phase ODS, and preparative HPLC, they were isolated from the rat urine sample, including seven new compounds, M-2, M-3, M-4, M-7, M-10, M-11, and M-12, together with six known alkaloids M-0, M-1, M-5, M-6, M-8, and M-9. Among the seven new compounds, M-10 (20.0 mg), M-3 (18.0 mg), and M-7 (15.0 mg) were three major metabolites, and M-11 (2.7 mg), M-12 (2.5 mg), M-4 (2.4 mg), and M-2 (2.1 mg) four minor ones.

Metabolite M-10 was isolated as a white amorphous powder. The molecular formula was determined to be C₂₁H₂₂N₂O₅ from the [M + H]⁺ quasi molecular ion peak at *m/z* 383.1607 (calcd. 383.1604) in the ESI-QTOF-MS. This implies that it has 12 degrees of unsaturation. The molecular weight of M-10 was the same as that of ICN (C₂₂H₂₆N₂O₄), but its molecular composition was different from that of ICN. ¹H-NMR (400 MHz, DMSO-*d*₆) spectra gave a group of *o*-substituted benzene ring proton signals (δ_{H} 7.22, 1H, o, δ_{H} 7.16, 1H, d, *J* = 7.0 Hz, δ_{H} 7.01, 1H, t, *J* = 7.2 Hz and δ_{H} 6.88, 1H, d, *J* = 7.6 Hz), which were in connection with four aromatic carbon signals (δ_{C} 128.5, 124.2, 121.6, and 109.9) in the HSQC spectra. ¹³C-NMR (100 MHz, DMSO-*d*₆) spectra gave a lactam carbonyl (δ_{C} 178.8), two aromatic quaternary carbons (δ_{C} 130.1 and δ_{C} 141.7), and a quaternary carbon (δ_{C} 50.7) signals. These data indicate that M-10 possesses an oxindole structure. Moreover, the resonances for the carbons and protons in the ¹H and ¹³C NMR as well as HSQC spectra included three methylene (δ_{H} 0.92, 1.75, δ_{C} 30.7; δ_{H} 2.47, 2.72, δ_{C} 40.7; and δ_{H} 2.63, 3.91, δ_{C} 44.6), two methines (δ_{H} 2.63, δ_{C} 36.5; δ_{H} 2.63, δ_{C} 40.8), a methane connected to N or O atom (δ_{H} 3.68, δ_{C} 61.7), a vinyl group (δ_{H} 4.99, 1H, d, *J* = 9.6 Hz, δ_{H} 5.02, 1H, d, *J* = 13.4 Hz, δ_{C} 116.3; δ_{H} 5.49, 1H, m, δ_{C} 138.2), a methoxy (δ_{H} 3.72, 3H, s, δ_{C} 61.3), an sp² methine (δ_{H} 7.23, 1H, s, δ_{C} 159.8) that should be directly attached to an oxygen, an sp² quaternary carbon (δ_{C} 110.5), and two carbonyls (δ_{C} 170.2 and 167.8). 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. The NMR data of M-10 (Table 1 and 2) suggested that its

tetracyclic framework was similar to that of ICN except that C-5 of M-10 was a carbonyl (δ_C 170.2). Moreover, the remaining molecular formula $C_6H_8O_3$ consisted structurally of two chain substituents, accounting for three degrees of unsaturation and being attached to the cyclic framework separately at C-15 and C-20. The constitution of one of the substituents, $C_4H_5O_3$, 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 the methoxy carbon, respectively. Subsequently, the remaining substituent, a vinyl group ($-CH=CH_2$), could be attached only to C-20, as evidenced by the appreciable correlation between H-18 and C-20 in the HMBC experiment of M-10. The absolute configuration of M-10 was determined from the CD spectrum and 2D NOESY correlation. As a tetracyclic oxindole alkaloid, its 7*S* configuration was established according to a negative CE at 285 nm and a positive CE at 220 nm in the CD spectrum [18,19]. Meanwhile, a negative CE at 265 nm indicated that H-3 had an α -orientation [19]. 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/ β -H-14 (δ_H 1.75). Thus, M-10 possesses 7*S*, 3*S*, 15*S*, 20*R* absolute configurations, which are consistent with the absolute configuration (*allo* A configuration) for ICN [19]. The geometry of the *trans* C-16–C-17 double bond was confirmed on the basis of the olefinic proton in a downfield shift relative to the corresponding signals of the *cis* compounds [20]. M-10 was determined to be 5-oxoisocorynoxenic acid.

Metabolite M-3 was isolated as a white amorphous powder. The molecular formula was determined to be $C_{27}H_{30}O_{11}$ from the $[M + H]^+$ quasi molecular ion peak at m/z 559.1928 (calcd. 559.1925) in the ESI-QTOF-MS. The $[M + H]^+$ ion at m/z 559 and an important fragment ion at m/z 383 originated from the elimination of 176 mass units (glucuronic acid) from $[M + H]^+$ ion indicated that M-3 should be a glucuronide conjugate. Its NMR data (Table 1 and 2) suggested that its tetracyclic framework was the same as that of M-10. The constitution and location of both substituents $C_4H_4O_3$ and C_2H_3 were also deduced from the HMBC spectrum. 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 -D-glucuronic acid moiety [21]. The correlation between an anomeric proton and C-22 in the HMBC experiment of M-3 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 (–2.5 ppm) relative to the corresponding signals of M-10. The full assignments of proton and carbon signals are summarized in Table 1 and 2. For M-3, the 7*S* 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 [18,19]. 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/ β -H-14. Thus, M-3 possesses 7*S*, 3*S*, 15*S*, 20*R* absolute configurations, which are consistent with the *allo* A configuration for ICN [19]. M-3 was determined to be 5-oxoisocorynoxenic acid-22-*O*- β -D-glucuronide.

Metabolite M-7 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 385.1763 (calcd. 385.1765) in the ESI-QTOF-MS. Its NMR data (Table 1 and 2) suggested that its

tetracyclic framework was the same as that of M-10. The constitution and location of both substituents $C_4H_7O_3$ and C_2H_3 were also deduced from the HMBC spectrum. For M-7, the 7*S* 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 [18,19]. The α -orientation 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 NO-ESY correlations of H-20/ β -H-14. Thus, M-7 possesses 7*S*, 3*S*, 15*S*, 16*S*, 20*R* absolute configurations, which are consistent with the *allo* A configuration for ICN [19]. M-7 was determined to be 17-*O*-demethyl-16,17-dihydro-5-oxoisocorynoxine.

Metabolite M-11 was isolated as a white amorphous powder. The molecular formula was determined to be $C_{22}H_{24}N_2O_6$ from the $[M + H]^+$ quasi molecular ion peak at m/z 413.1713 (calcd. 413.1714) in the ESI-QTOF-MS. Its NMR data (Table 1 and 2) suggested that its tetracyclic framework was similar to that of M-10 except that C-21 of M-11 was a methane connected to N or O atom (δ_H 5.35, 1H, t, $J = 3.5$ Hz, δ_C 73.7). According to the molecular formula, this implied that a hydroxyl group was attached to C-21 of M-11. The constitution and location of both substituents $C_5H_7O_3$ and C_2H_3 were also deduced from the HMBC spectrum. For M-11, the 7*S* 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 [18,19], as evidenced by the substantial NOE correlation of H-9/ β -H-14. The α -orientation of H-15 was indicated by the strong NOESY correlations of H-3/ α -H-14 and H-15/ α -H-14. The β -orientation of H-20 was indicated by the strong NOESY correlations of H-20/ β -H-14. The β -orientation of H-21 was confirmed on the basis of the correlations of H-21/ β -H-20 in the NOE data. Thus, metabolite M-11 possesses 7*S*, 3*S*, 15*S*, 20*R*, 21*R* absolute configurations, which are consistent with the *allo* A configuration for ICN [19]. M-11 was determined to be 21-hydroxy-5-oxoisocorynoxine.

Metabolite M-12 was isolated as a white amorphous powder. The molecular formula was determined to be $C_{22}H_{24}N_2O_6$ from the $[M + H]^+$ quasi molecular ion peak at m/z 413.1713 (calcd. 413.1716) in the ESI-QTOF-MS. Its NMR data (Table 1 and 2) suggested that its tetracyclic framework was the same as that of M-10. The resonances for the carbons and protons of both substituents $C_5H_7O_3$ and C_2H_3O in the 1H and ^{13}C NMR as well as HSQC spectra included an epoxide group (δ_H 2.57, 1H, dd, $J = 5, 1.5$ Hz, δ_C 52.3; δ_H 2.63, 1H, dd, $J = 5, 1.5$ Hz, δ_H 2.57, 1H, dd, $J = 5, 1.5$ Hz, δ_C 45.9), a carboxylic carbonyl (δ_C 167.0), two methoxyl groups (δ_H 3.75, 3H, s, δ_C 61.6; δ_H 3.59, 3H, s, δ_C 50.7), and an sp^2 methine (δ_H 7.30, s, δ_C 160.6) that should be directly attached to the oxygen. The constitution of one of the substituents, $C_5H_7O_3$, and its location at C-15 were deduced from the HMBC spectrum. The HMBC correlations of the substituents were determined between the one methoxyl proton (δ_H 3.75) and C-17 and between the other methoxyl proton (δ_H 3.59) and C-22 and between H-17 and C-15, C-16, and C-22, respectively. Subsequently, the remaining substituent, an epoxide group, C_2H_3O , could be attached only to C-20, as evidenced by the appreciable correlation between H-18 and C-20 in the HMBC experiment of M-12. For M-12, the 7*S* 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 [18,19], as evidenced by the substantial NOE correlation of H-9/ β -H-14. The α -orientation of H-15 was indicated by the strong NOESY correlations of H-3/ α -H-14 and H-15/ α -H-14. The β -orientation of H-20 was indicated by the strong NOESY correlations of H-20/ β -H-14. Thus, metabolite M-12 possesses 7*S*, 3*S*, 15*S*, 20*R* absolute configurations, which are consistent with the *allo* A configuration for ICN [19]. M-12 was determined to be oxireno [18,19]-5-oxoisocorynoxine.

Metabolite M-4 was isolated as a white amorphous powder. The molecular formula was determined to be C₂₂H₂₆N₂O₅ from the [M + H]⁺ quasi molecular ion peak at *m/z* 399.1920 (calcd. 399.1922) in the ESI-QTOF-MS which was 16 mass units higher than that of ICN. In the ¹H NMR spectrum, the skeleton proton signals (Table 1) of its parent drug remained except for those of the substituent group on the benzene ring. The linked positions of one hydroxyl group were established by the HMBC spectrum: a significant correlation between a proton signal at δ_{H} 6.79 (1H, d, $J_{9,11}$ = 2.5 Hz, H-9) and a carbon signal at δ_{C} 152.4 (C-10), or at δ_{C} 56.4 (C-7), indicated a hydroxyl group attached at C-10 in the aromatic ring. For M-4, the 7*S* 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 [18,19], as evidenced by the substantial NOE correlation of H-9/ β -H-14. The α -orientation of H-15 was indicated by the strong NOESY correlations of H-3/ α -H-14 and H-15/ α -H-14. The β -orientation of H-20 was indicated by the strong NOESY correlations of H-20/ β -H-14. Thus, metabolite M-4 possesses 7*S*, 3*S*, 15*S*, 20*R* absolute configurations. M-4 was identified as 10-hydroxyisocorynoxine.

Metabolite M-2 was isolated as a white amorphous powder. The molecular formula was determined to be C₂₂H₂₆N₂O₅ from the [M + H]⁺ quasi molecular ion peak at *m/z* 399.1920 (calcd. 399.1919) in the ESI-QTOF-MS. This implied that M-2 might be an isomer of M-4, which was further confirmed by NMR data (Table 1). A comparison of the ¹H NMR spectrum of M-2 with that of M-4 showed that their structures were closely related, except for the position of the hydroxyl group on the benzene ring. Then, according to protons signal at δ_{H} 7.22 (1H, d, $J_{9,10}$ = 8.0 Hz, H-9), 6.55 (1H, dd, $J_{9,10}$ = 8.0 Hz, $J_{10,12}$ = 1.5 Hz, H-10), and 6.69 (1H, d, $J_{10,12}$ = 1.5 Hz, H-12), we concluded that a hydroxyl group was attached to C-11 of M-2. For M-2, the absolute configurations of the asymmetric centers at C-3 and C-7 were assigned as *S* and *S*, respectively, by comparing its CD spectrum with those of IC and M-4. M-2 was identified as 11-hydroxyisocorynoxine.

The metabolites M-0, M-1, M-5, M-6, M-8 and M-9 were identified as isocorynoxine, 18,19-dehydrocorynoxinic acid, 18,19-dehydrocorynoxinic acid B, corynoxine, isocorynoxine-*N*-oxide, and corynoxine-*N*-oxide, respectively, by comparing their UV, MS, and *t_R* data with those of standard compounds. All of them were first isolated as metabolites of UH from rat urine.

The metabolic pathways of indole alkaloids *in vivo* are related to many reactions such as hydrolysis, *O*-demethylation [22], glucuronidation [13,14], sulfation [23,24], hydroxylation [25–27], reduction [15], and others [28]. In the present study, eleven phase I metabolites (M-1, M-2, and M-4–M-12), one phase II metabolite (M-3), and ICN (M-0) itself were isolated from the urine of rats given ICN orally and structurally confirmed on the basis of

UV, NMR, MS, and CD data. Also the neuroprotection of ICN and its metabolites, M-3, M-6, M-7, and M-10, against 3 mM glutamate-induced HT22 cell death was investigated. According to the metabolite profile, the possible metabolic pathways of ICN in rats are proposed as shown in Fig. 2.

M-3, M-7, and M-10 are three major metabolites. M-10 is believed to be formed through the oxidation at C-5 and hydrolysis of 22-carboxylic methyl ester catalyzed by hepatic cytochrome P450 enzyme [22,29–33]. Moreover, M-10 is also a reactive metabolite that can be successively converted to a glucuronide-conjugate (M-3) via further glucuronidation at 22-carboxylic group catalyzed by UDP-glucuronosyltransferases [22,34]. Most importantly, we found that M-7 is another key metabolite derived from the oxidation at C-5 and reduction of the 16-double bond together with the *O*-demethylation at C-17 catalyzed by cytochrome P450 [15,24,35]. Wang et al. [13,14] 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, hydroxylated at C-10 or C-11, derivatives of ICN were only found as minor metabolites in rat urine after oral administration of ICN 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 ICN, which is similar to the *in vivo* metabolism of nicotine [30,33] and (–)-N-{2-[(R)-3-(6,7-di-methoxy-1,2,3,4-tetrahydroisoquinoline-2-carbonyl)piperidino] ethyl}-4-fluorobenzamide [32]. 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-ICN in urine samples of rats orally given ICN dissolved in 0.1 M HCl solvent were lower than those in DMSO, while peak areas of 5-oxo-ICN in urine samples of rats orally given ICN 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, phytochemicals, such as tablets of *Uncaria rhynchophylla* total alkaloids, containing chloride salts of rhynchophyllinoid alkaloids have been used for several decades in clinical situations to treat hypertension, headache, and stroke in China. Thus, the oxidation at C-5 should be the major metabolic pathway for tetracyclic oxindole alkaloids, which is consistent with our last study of the metabolic fate of IRN in rats orally given IRN [36].

In addition, we also isolated some minor metabolites except for the parent compound ICN (M-0), all of which were phase I metabolites (M-1, M-2, M-4~M-6, M-8, M-9, M-11, and M-12). It is worth noting that the purity of the dosed ICN was found to be 98.6%, and that these minor metabolites were not impurities of the dosed ICN sample according to its UHPLC analysis. Formation of nine minor phase I metabolites can be explained by three phase I metabolic pathways, isomerization, oxidation and hydrolysis. Some of metabolic pathways mentioned above were firstly reported in the metabolism of tetracyclic oxindole alkaloids found in UH in rats after oral administration. The first is *N*-oxidation at N-4. M-8 and M-9 were two nitrogen oxides that are believed to be formed through the *N*-oxidation of N-4 under catalytic action of FMO₃ enzyme [33]. The second is epoxidation. M-12 is a

minor phase I metabolite derived from the epoxidation of the vinyl group at C-20 catalyzed by cytochrome P450 [37]. The last is the isomerization. Two pairs of epimers (M-0 and M-6, M-1 and M-5) were obtained. M-6 was the epimer of M-0 that is believed to be formed through the isomerization at C-7 in rats after the oral administration of ICN. IRN is reported to undergo isomerization at the *spiro* center to form RN in rat urine after oral administration of IRN by our last study [36]. In addition, the isomerization at the *spiro* centre of rhynchophylline-type oxindole alkaloids *in vitro* was recognized by Wenkert et al. as early as 1959 and a retro-Mannich ring opening, rotation, and Mannich ring closure was proposed as the mechanism involved. The isomerization at the *spiro* centre *in vivo* may occur without enzymatic conversion [38]. M-6 is also a reactive metabolite that can be successively converted to a hydrolyzed metabolite (M-5) via further hydrolysis of 22-carboxylic methyl ester. M-1 was the epimer of M-5 that is believed to be formed through the isomerization at C-20. There is, however, no definitive evidence for isomerization at C-20 of oxindole alkaloids *in vitro*. Thus, we postulated that isomerization at C-20 may be accomplished by suitable enzymatic processes *in vivo*.

Structural elucidation of metabolites is one of the most challenging tasks in drug metabolism studies. In recent years, comparisons of ESI-MSⁿ data and HPLC retention times with synthetic standards have been commonly used to identify the structures of metabolites. However, when the standards are difficult to synthesize, some metabolite structures deduced only from LC/MSⁿ data may not be correct, especially if isomeric metabolites are present. In our study, four pairs of isomers (M-0 and M-6, M-1 and M-5, M-2 and M-4, and M-8 and M-9) were obtained, which had identical MS data in the LC-MSⁿ determination. Therefore, their exact structures could not be identified using LC/MSⁿ data alone. In these cases, preparation of metabolites and further identification on the basis of NMR data are needed. Of course, direct isolation of the metabolites from urine, bile, or feces of humans or animals is difficult, but it is the most reliable method for the identification of metabolites. We have determined the definitive structures of 12 metabolites and the parent drug by examination of MS, CD, and NMR spectra. These results are important for better understanding of its *in vivo* metabolic fate and disposition in rats.

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 [39–41]. 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 [42,43]. The neuroprotective effects of some metabolites of ICN, together with ICN, against 3 mM glutamate-induced HT22 cell death were investigated. Cell viability was determined in HT22 cells by the calcein AM cell viability assay [42]. As shown in Fig. 3, ICN exhibited a significant neuroprotective effect against glutamate-induced cell death at the maximum concentration (100 μ M). In a parallel experiment, ZYC-26, an estrogen-like compound, produced a significant neuroprotective effect against glutamate-induced cell death [44]. However, little or weak neuroprotective activities were observed at the maximum concentration (100 μ M) for M-3, M-6 (CN), M-7, and M-10. The C-7 configuration of ICN and CN 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 7*S* configuration of the oxindole moiety. It was apparent that ICN 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, the reduction of the 16-double bond, *O*-demethylation at C-17, hydrolysis at 22-carboxylic methyl ester, and/or the 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, seven new together with six known compounds were isolated from the urine of rats given ICN orally and structurally confirmed by UV, NMR, MS, and CD techniques. It was evidenced for the first time that the major metabolic pathways for ICN should be the oxidation at C-5 and hydrolysis at the 22-carboxylic methyl ester. The formed metabolites were excreted either free or conjugated.

Materials and Methods

Chemical and reagents

ICN, CN, ICN-NO, CN-NO, 18,19-DCAB, and 18,19-DCA were isolated from the leaves of *Uncaria rhynchophylla* (Miq.) Miq. ex Havi., following our previously reported methods by Yuan et al. [8] and Ma et al. [45]. The identity of these compounds was confirmed by melting point, UV, IR, ¹H and ¹³C NMR, and MS. The purity of ICN evaluated with high-performance liquid chromatography was 98.6%, and those of others were more than 95%. ZYC-26 was a kind gift from Dr. James W. Simpkin [44] and selected as the positive drug for neuroprotective effects in the HT22 cell assay. The purity of ZYC-26 was 99.1%.

General experimental procedures

Macroporous resin AB-8 was obtained from Nankai University Chemical Factory; Sephadex LH-20 was from GE Healthcare; ODS was obtained from YMC Co., Ltd.; and silica gel GF₂₅₄ for thin-layer chromatography from Qindao Ocean Chemical Co., Ltd. Other chemical reagents were of analytical or HPLC grade. Double-distilled water from Jinzhou Scientific Instrument Co., Ltd. was used in this study.

Animals

Male Wistar rats (200 ± 20 g b.wt.) were purchased from the Animal Center of Shenyang Pharmaceutical University (approval date: 08/10/2012, No.: 100812, Shenyang, China). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Shenyang Pharmaceutical University. The animals 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.

Urine collection

Male Wistar rats were fasted 12 h before experiments. ICN (1.6 g) was given orally to 30 rats at a dose of 40 mg·kg⁻¹ (dissolved in 0.1 M HCl and diluted to 10 mg·mL⁻¹ with water, pH = 6.0 ~ 6.5) and administered repeatedly 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 .

Equipment

Spectroscopic methods—NMR spectra were measured on a Bruker ARX-300, 400, and 500 MHz spectrometer, and chemical shifts are given in ppm downfield relative to TMS as the internal standard. All compounds were dissolved in $\text{DMSO}-d_6$ or CDCl_3 . UV spectra were obtained using a Shimadzu UV-2201 spectrophotometer (Shimadzu). CD spectra were recorded on a Bio-Logic Modular Optical System 450 (MOS-450; Bio-Logic).

Q-TOF/MS^E parameters—Analyses were performed using a Micro-mass-Q-TOF Premier mass spectrometer (Waters Corp.) coupled with an ESI source operated in positive ion mode. 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 higher mass resolution. In the present study, the sensitivity mode was used for detection of some trace amount of metabolites in biological samples. The MS tune parameters were as follows: cone and desolvation gas flow were 50 L/h and 800 L/h, respectively; source temperature and desolvation were set at 130°C and 450°C , respectively; capillary and cone voltage were set at 3.0 kV and 40 eV, respectively; 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 DRE lock mass, a 2 ng/mL solution of leucine-enkephalin generating an $[\text{M} + \text{H}]^+$ ion (m/z 556.2771) was infused through the Lock Spray probe at 10 $\mu\text{L}/\text{min}$.

UHPLC conditions—Chromatographic separation was performed on a Waters Acquity UPLCTM system (Waters Corp.) using a Waters Acquity HSS C_{18} column (100 mm $\times$ 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 PLNO was used. A 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.

Cell culture

HT22 cells were cultured in DMEM (HyClone) supplemented with 10% FBS (Atlanta Biological) at 37°C in an atmosphere containing 5% CO_2 and 95% air [42]. HT22 cells were obtained from David Schubert (Salk Institute, USA).

Cell viability assay

Cell viability was determined in HT22 cells by the calcein AM cell viability assay [42]. In brief, HT22 cells were seeded 24 h before initiation of the experiment at a density of 5000 cells per well in 96-well plates and then treated with various test sample solutions with 3 mM glutamate which induced 50–75% cell death 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). Cells were incubated in a solution of 1 μ M calcein-AM in PBS at 37°C in dark. Twenty minutes later, fluorescence was determined using a Bio-Tek 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.

Extraction and isolation

The cumulative urine samples (approximately 3.6 liters in total) were thawed at room temperature and successively passed through a macroporous absorption resin AB-8 column eluting with a gradient of EtOH-H₂O (H₂O, 50% EtOH-H₂O, 70% EtOH-H₂O, and 95% EtOH-H₂O elutions) to yield four major fractions (Fr.1–4).

The 50% EtOH-H₂O fraction (Fr.2) was passed through a Sephadex LH-20 column (35 $\times$ 800 mm) and eluted with MeOH-H₂O (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 $\times$ 500 mm) eluting with MeOH-H₂O (50 :50) to yield one major fraction. Finally, pre-HPLC was carried out using MeOH-H₂O (15:85) plus 0.03% Et₂NH as an eluent at a flow rate of 1 mL·min⁻¹ to yield M-1 (1.4 mg), M-2 (2.1 mg), M-3 (18.0 mg), and M-4 (2.4 mg).

The 70% EtOH-H₂O (Fr.3) fraction was passed through a Sephadex LH-20 column (35 $\times$ 800 mm) chromatography and a linear gradient of MeOH-H₂O (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 M-5 (1.3 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 M-6 (2.4 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 M-7 (2.3 mg), M-8 (15.0 mg), M-9 (2.5 mg), and M-10 (2.1 mg). Fraction (Fr.3–3) was further separated through an ODS open column eluting with a gradient of MeOH-H₂O (50:50) at a flow rate of 1 mL·min⁻¹ to yield M-10 (20.0 mg) and M-11 (2.7 mg).

The 95% EtOH fraction (Fr.4) was further separated by a Sephadex LH-20 column (35 $\times$ 800 mm) and eluted with MeOH-H₂O (70:30) system and a pre-HPLC eluting with MeOH-H₂O (65:35) plus 0.03% Et₂NH to yield M-12 (2.5 mg).

Statistical methods

Statistical significance was determined by one-way analysis of variance (ANOVA) using the SPSS 11.5 software package. Results are expressed as mean $\pm$ SEM for $n = 8$. All data were normalized to percentage survival of control. Dunnett's test was used to determine whether the percentage survival of various test sample solutions differed significantly from the control. Statistical significance was set at $p < 0.05$ level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (NSFC) (No. 81173544) and the Distinguished Professor Foundation of Liaoning Province of China of 2011.

Abbreviations

CE	Cotton effect
CN	corynoxine
CN-NO	corynoxine- <i>N</i> -oxide
18, 19-DCA	18,19-dehydrocorynoxinic acid
18,19-DCAB	18,19-dehydrocorynoxinic acid B
DRE	dynamic range enhancement
G	glutamate
ICN	isocorynoxine
ICN-NO	isocorynoxine- <i>N</i> -oxide
IRN	isorhynchophylline
M-0	isocorynoxine
M-1	18,19-dehydrocorynoxinic acid
M-2	11-hydroxyisocorynoxine
M-3	5-oxoisocorynoxineic acid-22- <i>O</i> - β -D-glucuronide
M-4	10-hydroxyisocorynoxine
M-5	18,19-dehydrocorynoxinic acid B
M-6	corynoxine
M-7	17- <i>O</i> -demethyl-16,17-dihydro-5-oxoisocorynoxine
M-8	isocorynoxine- <i>N</i> -oxide
M-9	corynoxine- <i>N</i> -oxide

M-10	5-oxoisocorynoxenic acid
M-11	21-hydroxy-5-oxoisocorynoxene
M-12	oxireno[18,19]-5-oxoisocorynoxene
MCP	micro-channel plate
PLNO	partial loop using needle overfill mode
RN	rhynchophylline
TMS	tetramethylsilane
UH	Uncaria hook
ZYC-26	2-(1-adamantyl)-4-methylestrone

References

1. Qu JL, Gong TX, Ma B, Zhang L, Kano Y, Yuan D. Comparative study of fourteen alkaloids from *Uncaria rhynchophylla* hooks and leaves using HPLC-diode array detection-atmospheric pressure chemical ionization/MS method. *Chem Pharm Bull.* 2012; 60:23–30. [PubMed: 22223371]
2. Shi JS, Liu GX, Wu Q, Huang YP, Zhang XD. Effects of rhynchophylline and isorhynchophylline on blood pressure and blood flow of organs in anesthetized dogs. *Zhongguo Yao Li Xue Bao.* 1992; 13:35–38. [PubMed: 1605032]
3. Zhang WB, Chen CX, Sim SM, Kwan CY. *In vitro* vasodilator mechanisms of the indole alkaloids rhynchophylline and isorhynchophylline, isolated from the hook of *Uncaria rhynchophylla* (Miquel). *Naunyn Schmiedeberg Arch Pharmacol.* 2004; 369:232–238. [PubMed: 14668978]
4. Kang TH, Murakami Y, Takayama H, Kitajima M, Aimi N, Watanabe H, Matsumoto K. Protective effect of rhynchophylline and isorhynchophylline on *in vitro* ischemia-induced neuronal damage in the hippocampus: putative neurotransmitter receptors involved in their action. *Life Sci.* 2004; 76:331–343. [PubMed: 15531384]
5. Sakakibara I, Terabayashi S, Kubo M, Higuchi M, Kamatsu Y, Okada M, Taki K, Kamei J. Effect on locomotion of indole alkaloids from the hooks of *Uncaria* plants. *Phytomedicine.* 1999; 6:163–168. [PubMed: 10439480]
6. Li JJ, Tang JL, Hu LL, Zhou SW, Zhou JY. Effect of rhynchophylla total alkaloids on behavior and contents of monoamine neurotransmitters in brain tissues of anxiety model rats. *Third Mil Med Univ.* 2013; 35:237–240.
7. Shimada Y, Goto H, Itoh T, Sakakibara I, Kubo M, Sasaki H, Terasawa K. Evaluation of the protective effects of alkaloids isolated from the hooks and stems of *Uncaria sinensis* on glutamate-induced neuronal death in cultured cerebellar granule cells from rats. *Pharm Pharmacol.* 1999; 51:715–722.
8. Yuan D, Ma B, Wu CF, Yang JY, Zhang LJ, Liu SK, Wu LJ, Kano Y. Alkaloids from the leaves of *Uncaria rhynchophylla* and their inhibitory activity on NO production in lipopolysaccharide-activated microglia. *J Nat Prod.* 2008; 71:1271–1274. [PubMed: 18588343]
9. Matsumoto K, Morishige R, Murakami Y, Tohda M, Takayama H, Sakakibara I, Watanabe H. Suppressive effects of isorhynchophylline on 5-HT_{2A} receptor function in the brain: behavioural and electrophysiological studies. *Eur J Pharmacol.* 2005; 517:191–199. [PubMed: 15963493]
10. Huang MJ, Hao JC, Wang JL, Wang W. Biotransformation of corynoxene and isocorynoxene in rats. *J Kunming Medical University.* 2012; 10:26–30.
11. Cai JZ, Lin CL, Hu LF, Lin GY, Wang XQ, Ma JS. Determination of isocorynoxene in rat plasma by liquid chromatography mass spectrometry and its application. *J Liq Chromatogr Relat Technol.* 2013; 36:2232–2241.

12. Wang W, Li XM, Chen YP, Hattori M. Structural elucidation of rat biliary metabolites of corynoxine and their quantification using LC-MSⁿ. *Biomed Chromatogr.* 2014; 28:1219–1228. [PubMed: 24523045]
13. Wang W, Ma CM, Hattori M. Metabolism and pharmacokinetics of rhynchophylline in rats. *Biol Pharm Bull.* 2010; 33:669–676. [PubMed: 20410604]
14. Wang W, Ma CM, Hattori M. Metabolism of isorhynchophylline in rats detected by LC-MS. *J Pharm Pharm Sci.* 2010; 13:27–37. [PubMed: 20456828]
15. Yu JX, Xie XL, Wu Q, Huang XN, Sun AS, Shi JS. Mass spectroscopic identification and pharmacological effect of the metabolite of isorhynchophylline. *Chin Remed Clin.* 2005; 5:7–10.
16. Yasuda T, Ohsawa K. Urinary metabolites of daidzin orally administered in rats. *Biol Pharm Bull.* 1998; 21:953–957. [PubMed: 9781846]
17. Bursztyka J, Perdu E, Tulliez J, Debrauwer L, Delous G, Canlet C, De Sousa G, Rahmani R, Benfenati E, Cravedi JP. Comparison of genistein metabolism in rats and humans using liver microsomes and hepatocytes. *Food Chem Toxicol.* 2008; 46:939–948. [PubMed: 18063284]
18. Trager WF, Lee CM, Phillipson JD, Haddock RE, Dwuma-Badu D, Beckett AH. Configurational analysis of rhynchophylline-type oxindole alkaloids. The absolute configuration of ciliaphylline, rhynchociline, specionoxine, isospecionoxine, rotundifoline and isorotundifoline. *Tetrahedron.* 1969; 24:523–543. [PubMed: 5637484]
19. Phillipson JD, Hemingway SR. Alkaloids from *Uncaria* species. III. Oxindole alkaloids from *Uncaria macrophylla*. *Phytochem.* 1973; 12:2795–2798.
20. Lee CM, Trager WF, Beckett AH. Corynantheidine-type alkaloids-II: Absolute configuration of mitragynine, speciociliatine, mitraciliatine and speciogynine. *Tetrahedron.* 1967; 23:375–385. [PubMed: 6037287]
21. Yasuda T, Kano Y, Saito K, Ohsawa K. Urinary and biliary metabolites of daidzin and daidzein in rats. *Biol Pharm Bull.* 1994; 17:1369–1374. [PubMed: 7874061]
22. Philipp AA, Wissenbach DK, Weber AA, Zapp J, Maurer HH. Metabolism studies of the Kratom alkaloids mitraciliatine and isopaynantheine, diastereomers of the main alkaloids mitragynine and paynantheine, in rat and human urine using liquid chromatography-linear ion trap-mass spectrometry. *J Chromatogr B.* 2011; 879:1049–1055.
23. Vigano V, Paracchini S, Piacenza G, Pesce E. Metabolism of vincamine in the rat. *Farmaco Sci.* 1978; 33:583–594. [PubMed: 744256]
24. Zhao T, Zheng SS, Zhang BF, Li YY, Bligh ASW, Wang CH, Wang ZT. Metabolic pathways of the psychotropic-carboline alkaloids, harmaline and harmine, by liquid chromatography/mass spectrometry and NMR spectroscopy. *Food Chemistry.* 2012; 134:1096–1105. [PubMed: 23107733]
25. Corre PL, Dollo G. Biopharmaceutics and metabolism of yohimbine in humans. *Eur J Pharm Sci.* 1999; 9:79–84. [PubMed: 10494000]
26. Ueng YF, Yu HJ, Lee CH, Peng C, Jan WC, Ho LK, Chen CF, Don MJ. Identification of the microsomal oxidation metabolites of rutaecarpine, a main active alkaloid of the medicinal herb *Evodia rutaecarpa*. *J Chromatogr A.* 2005; 1076:103–109. [PubMed: 15974075]
27. Nakazawa T, Banba K, Hata K, Nihei Y, Hoshikawa A, Ohsawa K. Metabolites of hirsuteine and hirsutine, the major indole alkaloids of *Uncaria rhynchophylla*, in rats. *Biol Pharm Bull.* 2006; 29:1671–1677. [PubMed: 16880624]
28. Vereczkey L, Tamás J, Czira G, Szporny L. Metabolism of vincamine in the rat *in vivo* and *in vitro*. *Arzneimittelforschung.* 1980; 30:1860–1865. [PubMed: 7192994]
29. Wu WN, McKown LA. The *in vitro* metabolism of thalicarpine, an aporphine–benzyltetrahydroisoquinoline alkaloid, in the rat API-MS/MS identification of thalicarpine and metabolites. *J Pharmaceut Biomed.* 2002; 30:141–150.
30. Tyroller S, Zwickenpflug W, Thalheim C, Richter E. Acute and subacute effects of tobacco alkaloids, tobacco-specific nitrosamines and phenethyl isothiocyanate on *N*-nitrosonornicotine metabolism in rats. *Toxicology.* 2005; 215:245–253. [PubMed: 16118032]
31. Zhang HG, Sun Y, Duan MY, Chen YJ, Zhong DF, Zhang HQ. Separation and identification of *Aconitum* alkaloids and their metabolites in human urine. *Toxicon.* 2005; 46:500–506. [PubMed: 16135377]

32. Umehara K, Wada K, Noguchi K, Iwatsubo T, Usui T, Hidetaka K. Relationship between exposure of (–)-N-[2-[(R)-3-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline-2-carbonyl)piperidino]ethyl]-4-fluorobenzamide (YM758), a “funny” if current channel inhibitor, and heart rate reduction in tachycardia-induced beagle dogs. *Drug Metab Dispos.* 2009; 37:1427–1433. [PubMed: 19359407]
33. Miller EI, Norris HK, Rollins DE, Tiffany ST, Wilkins DG. A novel validated procedure for the determination of nicotine, eight nicotine metabolites and two minor tobacco alkaloids in human plasma or urine by solid-phase extraction coupled with liquid chromatography-electrospray ionization-tandem mass spectrometry. *J Chromatogr B.* 2010; 878:725–737.
34. Lee YP, Hsu FL, Kang JJ, Chen CK, Lee SS. Metabolism of (2S)-pterosin A: identification of the phase I and phase II metabolites in rat urine. *Drug Metab Dispos.* 2012; 40:1566–1574. [PubMed: 22587987]
35. Zeng Y, Qiu F, Liu Y, Qu G, Yao X. Isolation and identification of phase I metabolites of demethoxycurcumin in rats. *Drug Metab Dispos.* 2007; 35:1564–1573. [PubMed: 17553917]
36. Chen FF, Qi W, Sun JH, Simpkins JW, Yuan D. Urinary metabolites of isorhynchophylline in rats and their neuroprotective activities in the HT22 cell assay. *Fitoterapia.* 2014; 97:156–163. [PubMed: 24910000]
37. Eichelbaum M, Tomion T, Tybring G, Bertilsson L. Carbamazepine metabolism in man: induction and pharmacogenetic aspects. *Clin Pharmacokinet.* 1985; 10:80–90. [PubMed: 3971637]
38. Wenkert E, Udelhofen JH, Bhattacharyya NK. 3-Hydroxymethyleneoxindole and its derivatives. *J Am Chem Soc.* 1959; 81:3763–3768.
39. Corre PL, Dollo G. Biopharmaceutics and metabolism of yohimbine in humans. *Eur J Pharm Sci.* 1999; 9:79–84. [PubMed: 10494000]
40. Blandini F, Porter RH, Greenamyre JT. Glutamate and Parkinson’s disease. *Mol Neurobiol.* 1996; 12:73–94. [PubMed: 8732541]
41. Cherubinia A, Ruggiero C, Polidorib MC, Mecoccia P. Potential markers of oxidative stress in stroke. *Free Radic Biol Med.* 2005; 39:841–852. [PubMed: 16140205]
42. Yi KD, Chung J, Pang P, Simpkins WJ. Role of protein phosphatases in estrogen-mediated neuroprotection. *J Neurosci.* 2005; 25:7191–7198. [PubMed: 16079401]
43. Tan S, Wood M, Maher P. Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. *J Neurochem.* 1998; 71:95–105. [PubMed: 9648855]
44. Simpkins JW, Yang SH, Liu R, Perez E, Cai ZY, Covey DF, Green PS. Estrogen-like compounds for ischemic neuroprotection. *Stroke.* 2004; 35:2648–2651. [PubMed: 15472107]
45. Ma B, Wu CF, Yang JY, Wang R, Kano Y, Yuan D. Three new alkaloids from the leaves of *Uncaria rhynchophylla*. *Helv Chim Acta.* 2009; 92:1575–1585.

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

Fig. 2.
Proposed metabolic pathways of isocorynoxine.

Fig. 3. Effects of isocorynoxene and some metabolites against 3 mM glutamate-induced HT22 cell death. HT22 cells were treated simultaneously with 3 mM glutamate. Cell viability was determined by calcein AM assay after 24 h exposure to the various samples. All data were normalized to percentage survival of control. 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. CN: corynoxene; G: glutamate; ICN: isocorynoxene; ZYC-26, 2-(1-adamantyl)-4-methylestrone.

Table 1

¹H NMR data of ICN metabolites M-2~M-4, M-7, and M-10~M-12.

No.	M-2 ^a	M-3 ^b	M-4 ^a	M-7 ^c	M-10 ^d	M-11 ^a	M-12 ^a
2							
3	2.18, d (9.5)	3.69, d (8.4)	2.22, d (9.5)	3.82, dd α (3.6,12.0)	3.68, o	4.00, dd α (5.0, 12.0)	3.67, dd α (3.5,12.0)
5	2.37, o α		2.29, t α (8.5)				
	3.32, o β		3.20, td β (8.5, 2.5)				
6	1.81, o β	2.41, o β	1.89, td β (7.5, 2.5)	2.55, d β (16.4)	2.47, d β (16.8)	2.35, d β (17.0)	2.41, o β
	2.14, o α	2.72, d α (16.5)	2.15, o α	3.03, d α (16.4)	2.72, d α (16.8)	2.72, d α (15.5)	2.72, o α
7							
8							
9	7.22, d (8.0)	7.18, d (7.5)	6.79, d (2.5)	7.19, d (7.2)	7.16, d (7.0)	7.11, d (7.5)	7.11, d (7.5)
10	6.55, dd (1.5, 8.0)	7.01, t (7.5)		7.06, t (7.5)	7.01, t (7.2)	7.02, t (7.5)	7.00, td (7.5, 3.0)
11		7.21, t (7.5)	6.52, dd (2.0, 8.0)	7.28, o	7.22, o	7.22, t (7.5)	7.21, t (7.5)
12	6.69, d (1.5)	6.88, d (7.8)	6.58, d (8.0)	6.94, d (7.6)	6.88, d (7.6)	6.87, d (7.5)	6.88, d (8.0)
13							
14	0.84, m α	0.94, m α	0.83, m α	1.04, m β	0.92, m α	0.92, m α	0.92, m α
	1.43, m β	1.71, m β	1.42, m β	1.32, m α	1.75, m β	2.63, m β	1.66, m β
15	2.36, o	2.65, o	2.35, o	1.72, t (11.6)	2.63, o	3.16, d (5.0)	2.75, o
16			2.85, m				
17	7.26, s	7.39, s	7.22, s	3.54, s	7.23, s	7.21, s	7.30, s
				3.75, dd (7.6, 11.2)			
18	4.87, d (10.5)	4.97, d (10.5)	4.88, d (10.0)	5.23, o	4.99, d (9.6)	4.93, d (9.2)	2.28, m
	4.90, d (14.0)	5.07, d (17.4)	4.90, d (12.5)	5.26, d (10.0)	5.02, d (13.4)	4.93, d (9.2)	2.63, dd (1.5, 5.0)
19	5.44, m	5.43, m	5.45, m	5.55, m	5.49, m	5.52, m	2.57, dd (1.5, 5.0)
20	2.71, m	2.65, o	2.74, m	2.26, m	2.63, o	2.62, t (5.0)	1.65, m
21	1.89, o α	2.63, o α	1.82, o α	2.62, t α (12.0)	2.63, o α	5.35, t (3.5)	2.74, o α
	3.27, d β (12.5)	3.92, d β (7.8)	3.07, m β (12.5)	4.16, d β (9.2)	3.91, d β (9.2)		4.03, d β (12.75)
22							

No.	M-2 ^a	M-3 ^b	M-4 ^a	M-7 ^c	M-10 ^d	M-11 ^a	M-12 ^a
17-OCH ₃	3.73, s	3.78, s	3.72, s		3.72, s	3.72, s	3.75, s
22-OCH ₃	3.50, s		3.49, s	3.51, s		3.47, s	3.59, s
NH	9.80, br, s	10.58, br, s	10.02, br, s	8.70, br, s	10.59 br, s	10.58, br, s	10.56, br, s
1'	5.19, d, (7.5)						
2'	3.19, o						
3'	3.21, o						
4'	3.10, o						
5'	3.29, d (9.6)						
6'							

s: singlet; d: doublet; t: triplet; m: multiplet; o: overlapped;

^aMeasured separately at 500 MHz in DMSO;

^bMeasured separately at 300 MHz in DMSO;

^cMeasured separately at 400 MHz in CDCl₃;

^dMeasured separately at 400 MHz in DMSO

Table 2

¹³C NMR data of ICN metabolites M-2~M-4, M-7, and M-10~M-12.

No.	M-2 ^a	M-3 ^b	M-4 ^a	M-7 ^c	M-10 ^d	M-11 ^a	M-12 ^a
2	181.1, qC	178.5, qC	179.7, qC	179.1, qC	178.8, qC	178.2, qC	178.6, qC
3	71.9, CH	61.6, CH	71.4, CH	62.3, CH	61.7, CH	57.8, CH	61.4, CH
5	54.7, qC	170.2, qC	53.2, qC	171.2, qC	170.2, qC	170.8, qC	170.2, qC
6	34.7, CH ₂	41.1, CH ₂	35.6, CH ₂	41.5, CH ₂	40.7, CH ₂	41.6, CH ₂	40.8, CH ₂
7	56.4, qC	51.0, qC	56.4, qC	52.0, qC	50.7, qC	51.2, qC	50.8, qC
8	130.7, qC	130.2, qC	134.7, qC	130.3, qC	130.1, qC	130.3, qC	130.1, qC
9	124.2, CH	124.0, CH	112.2, CH	124.2, CH	124.2, CH	123.8, CH	124.1, CH
10	111.2, qC	122.0, CH	152.4, qC	122.7, CH	121.6, CH	121.7, CH	121.6, CH
11	151.8, qC	128.4, CH	113.3, CH	129.0, CH	128.5, CH	128.5, CH	128.5, CH
12	100.2, CH	109.9, CH	109.3, CH	110.6, CH	109.9, CH	110.0, CH	109.9, CH
13	141.6, qC	141.6, qC	133.1, qC	140.4, qC	141.7, qC	141.5, qC	141.7, qC
14	28.8, CH ₂	30.6, CH ₂	28.9, CH ₂	28.8, CH ₂	30.7, CH ₂	39.2, CH ₂	30.3, CH ₂
15	39.5, CH	36.0, CH	37.2, CH	39.3, CH	36.5, CH	30.2, CH	34.4, CH
16	111.5, qC	110.0, qC	110.6, qC	47.9, CH	110.5, qC	110.2, qC	109.3, qC
17	159.8, CH	161.2, CH	159.8, CH	61.6, CH ₂	159.8, CH	160.2, CH	160.6, CH
18	114.2, CH ₂	116.9, CH ₂	115.3, CH ₂	119.5, CH ₂	116.3, CH ₂	116.4, CH ₂	45.9, CH ₂
19	139.4, CH	137.9, CH	139.5, CH	136.7, CH	138.2, CH	138.3, CH	52.3, CH
20	41.9, CH	40.4, CH	41.4, CH	43.2, CH	40.8, CH	47.1, CH	39.1, CH
21	61.5, CH ₂	44.8, CH ₂	58.2, CH ₂	45.5, CH ₂	44.6, CH ₂	73.7, CH	42.5, CH
22	168.2, qC	165.3, qC	167.1, qC	173.3, qC	167.8, qC	167.0, qC	167.0, qC
17-OCH ₃	61.5, CH ₃	61.6, CH ₃	61.3, CH ₃		61.3, CH ₃	61.4, CH ₃	61.6, CH ₃
22-OCH ₃	51.3, CH ₃		50.6, CH ₃	51.3, CH ₃		50.7, CH ₃	50.7, CH ₃
NH							
1'		93.8, CH					
2'		72.2, CH					
3'		76.5, CH					

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

No.	M-2 ^a	M-3 ^b	M-4 ^a	M-7 ^c	M-10 ^d	M-11 ^a	M-12 ^a
4'	71.9, CH						
5'	74.3, CH						
6'	172.0						

^a Measured separately at 125 MHz in DMSO;
^b Measured separately at 75 MHz in DMSO;
^c Measured separately at 100 MHz in CDCl₃;
^d Measured separately at 100 MHz in DMSO