Future Integrative Medicine **2024** vol. 3(3) | 153–159
DOI: 10.14218/FIM.2024.00029

Original Article

Comparative Metabolism of the Humantenirine in Liver Microsomes from Pigs, Goats, and Humans

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Received: June 13, 2024 | **Revised:** August 03, 2024 | **Accepted:** August 15, 2024 | **Published online:** September 05, 2024

Abstract

Background and objectives: *Gelsemium elegans* Benth (*G. elegans*) is a traditional medicinal plant; however, it is highly toxic, and toxicity varies significantly between species. The cause of this difference has not been clarified. Humantenirine is an important toxic alkaloid in *G. elegans*, and its metabolism has been poorly studied. This study aimed to compare the different metabolites formed by human liver microsomes, pig liver microsomes, and goat liver microsomes.

Methods: High-performance liquid chromatography/quadrupole time-of-flight mass spectrometry was used to study the metabolism of humantenirine in human liver microsomes, pig liver microsomes, and goat liver microsomes.

Results: A total of eight metabolites (M1-M8) were identified, and three major metabolic pathways were found: demethylation (M1), dehydrogenation (M2, M3, M7), and oxidation (M4, M5, M6, M8).

Conclusions: Based on these results, it is hypothesized that demethylation is the major detoxification pathway for humantenirine, providing important information to better understand the metabolism and toxicity differences between species of *G. elegans*.

Introduction

Gelsemium elegans Benth (*G. elegans*) is an evergreen woody vine widely distributed in southern China. *G. elegans* is often used as a Chinese veterinary herb and can increase the appetite of pigs. Modern pharmacological studies have shown that *G. elegans* has multiple pharmacological effects, including immune regulation, anxiety reduction, anti-tumor properties, and neurological analgesia. However, *G. elegans* is also a toxic plant with strong neurotoxicity, particularly towards humans and rats. Oral gavage of *G. elegans* at a dose of 1 g/kg did not cause toxicity in pigs and goats but caused acute toxic deaths in rats.[1] *G. elegans* poisoning is characterized by nausea, vomiting, convulsions, and severe respiratory depression.[2,3] In recent years, poisoning incidents have occurred sporadically,[4] limiting its clinical use.

Keywords: Comparative metabolism; Humantenirine; *Gelsemium elegans*; Human; Mass spectrometry; Liver microsomes.

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How to cite this article: Wang Y, Qi X, Zuo M, Liu Z. Comparative Metabolism of the Humantenirine in Liver Microsomes from Pigs, Goats, and Humans. *Future Integr Med* 2024;3(3):153–159. doi: 10.14218/FIM.2024.00029.

Many compounds have been identified from *G. elegans*, including alkaloids, cyclic ether compounds, and steroids. The main active ingredients and toxic components of *G. elegans* are alkaloids.[5] Based on their structural characteristics, these alkaloids are classified into six categories: gelsenicine-type, humantenine-type, gelsemine-type, koumine-type, sarpagine-type, and yohimbane-type. Previous studies have reported on the pharmacokinetics and tissue distribution of koumine,[6,7] gelsemine,[8] and gelsenicine,[9] with demethylation, oxidation, and dehydrogenation being common metabolic pathways for all three alkaloids. The humantenine-type alkaloids include 28 compounds such as humantenine, humantendine, humantenirine, and rankinidine.[10] Among these, humantenine-type alkaloids are the main toxic compounds in *G. elegans* due to their very low median lethal dose (LD_{50}). For example, the LD_{50} of humantendine in mice is only 0.21 mg/kg,[11] and the LD_{50} of humantenirine in mice is only 0.071 mg/kg.[12] In previous studies by our research group, the metabolism of humantenine and rankinidine was investigated in mice, goats, pigs, and human liver microsomes, including the analysis of metabolism and tissue distribution after humantenirine administration in mice.[11,13,14] However, there has been no thorough study of humantenirine's metabolic profile in human liver microsomes (HLM), pig liver microsomes (PLM), and goat liver microsomes (GLM) until now. As humantenirine is a major toxicant in *G. elegans*, differences

in its metabolism are likely to be responsible for species-specific differences in *G. elegans* toxicity. Therefore, the present study is the first to investigate the metabolism of humantenirine in HLM, PLM, and GLM.

High-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (HPLC/QqTOF-MS) is widely used in the identification of Chinese drugs and metabolites in biological samples due to its high sensitivity and specificity.[15,16] By comparing the molecular mass, molecular formula, and MS2 spectrum between the prototype and metabolites, the structure of metabolites can be characterized quickly and reliably. Therefore, this study uses HPLC/QqTOF-MS to clarify the main metabolic pathways of humantenirine and to compare humantenirine metabolites in liver microsomes of different species. This study contributes to understanding the reasons for species-specific differences in *G. elegans* toxicity and lays the foundation for the development and application of *G. elegans*.

Materials and methods

Chemicals

Humantenirine was purchased from Chengdu Mansite Biotechnology Co. (Chengdu, Sichuan, China). The BCA Protein Assay Kit was obtained from ComWin Biotech Co., Ltd. (Beijing, China). Nicotinamide adenine dinucleotide phosphate (NADPH) was sourced from Beijing Solarbio Technology Co., Ltd. (Beijing, China). Formic acid and acetonitrile were purchased from Merck (Darmstadt, Germany). Ultrapure water was acquired using Milli-Q purification technology (Millipore, Bedford, MA, USA). All other chemicals and reagents used in this study were of high analytical grade and commercially available.

HLM were obtained from PrimeTox Biomedical Technology Co., Ltd (Wuhan, Hubei, China; 20 mg/mL pooled Chinese male donors), and the study conformed to the ethical guidelines of the Helsinki Declaration (as revised in 2013). Our earlier study described the sources of goats and pigs and the procedure for preparing liver microsomes from them[17]; before use, all liver microsomes were preserved at −70°C.

Metabolism of humantenirine in liver microsomes

The incubation mixture contained 10 mmol/L humantenirine, 5 mmol/L $MgCl_2$, 1 mg/mL liver microsome protein, 2 mmol/L NADPH, and 0.05 mol/L Tris-HCl buffer (pH 7.4) in a total volume of 200 μL. The reaction mixture was preincubated for 5 m at 37°C, and then the reaction was initiated by the addition of humantenirine. Control incubations were conducted in the absence of NADPH. After one hour, the reaction was terminated by adding 15% trichloroacetic acid (50 μL) to the mixture. The mixture was kept on ice until it was centrifuged for 15 m at 12,000 g, followed by filtration through a 0.22 μm membrane, and subsequent analysis under high-performance liquid chromatography/quadrupole time-of-flight (LC-QTOF) mass spectrometry analytical conditions. All incubations were performed in independent experiments, each conducted in triplicate.

LC-QTOF analytical conditions

The mass spectrometer operated in positive mode using an electrospray ionization source. Mass spectrometric analyses were carried out using full-scan MS mode with a mass range of m/z 50–1,000 and automatic MS/MS acquisition. The operating parameters were as follows: gas temperature, 300°C; capillary voltage, 4.0 kV; nebulizer pressure, 35 psi; sheath gas temperature, 350°C; sheath gas flow rate, 11 L/m; skimmer voltage, 65 V; and fragmentor voltage, 175 V. Nine liters of nitrogen per minute was used as the nebulizing gas. For mass corrections, automated calibrant delivery systems were used to provide accurate mass measurements for each peak from the total ion chromatograms. Agilent Mass Hunter software (B.01.03) was used to acquire the data. Methods for analyzing humantenirine metabolites have been described in previous studies.[14]

Results

Metabolites and metabolic pathways of humantenirine in PLM, GLM, and HLM

HPLC/QqTOF-MS analysis was performed to identify the metabolites of humantenirine in GLM, HLM, and PLM. A total of eight humantenirine metabolites (M1–M8) were identified across the three liver microsomes. Seven metabolites (M1–M7) were observed in HLM, six metabolites (M1–M3, M5, M6, and M8) were found in PLM, and seven metabolites (M1–M6, M8) were identified in GLM. The m/z of protonated molecular ions ([M+H]+) for metabolites M1–M8 were 357.1809, 369.1809, 369.1809, 387.1914, 387.1914, 385.1758, 385.1758, and 373.1758, respectively. Figure 1 shows the extracted ion chromatograms of the humantenirine metabolites.

In an experiment involving the oral administration of humantenirine to rats, a total of eight humantenirine metabolites were identified, including seven metabolites found in this study (M1–M3, M5–M8). Their retention times, elemental compositions, observed masses, predicted masses, mass errors, and other key information have been described in detail in previous papers.[14]

M4 is a newly discovered metabolite in HLM and GLM; however, M4 was not identified in PLM. The secondary mass spectrum was used to further analyze the structure of the metabolic product M4. Figure 2 shows the MS^2 map of the metabolites.

The chemical formula of metabolite M4 is $C_{21}H_{26}N_2O_5$, with a retention time of 9.8 m. A [M+H]+ ion of M4 at m/z 387, which was 16 Da higher than humantenirine, was observed. The MS^2 spectra of protonated M4 showed that the ions at m/z 341 and 180 were 16 Da higher than humantenirine at m/z 325 and 164, respectively. Therefore, it is speculated that M4 is an oxidation metabolite of humantenirine. Table 1 provides the molecular formula, accurate quality, and quality measurement error of humantenirine and its metabolites.

This study is the first to use HPLC/QqTOF-MS to identify the metabolites of humantenirine in PLM, HLM, and GLM. Based on the metabolic pathways of humantenirine,[18,19] as reported in previous studies, it is speculated that the possible metabolic pathways and metabolites of humantenirine involve three main processes: demethylation (M1), dehydrogenation (M2, M3, M7), and oxidation (M4, M5, M6, M8). Figure 3 illustrates the possible metabolic pathways of humantenirine in HLM, PLM, and GLM.

The relative peak intensities of humantenirine metabolites in different species

By comparing the relative peak intensities of these metabolites, among the eight metabolites, M1–M3, M5, and M6 were detected in all three liver microsomes, but the levels of these metabolites showed significant differences. In the liver microsomes of the three species, the content of M1 followed this order: pig > goat > human. Compared with HLM, the peak intensity of M1 in PLM

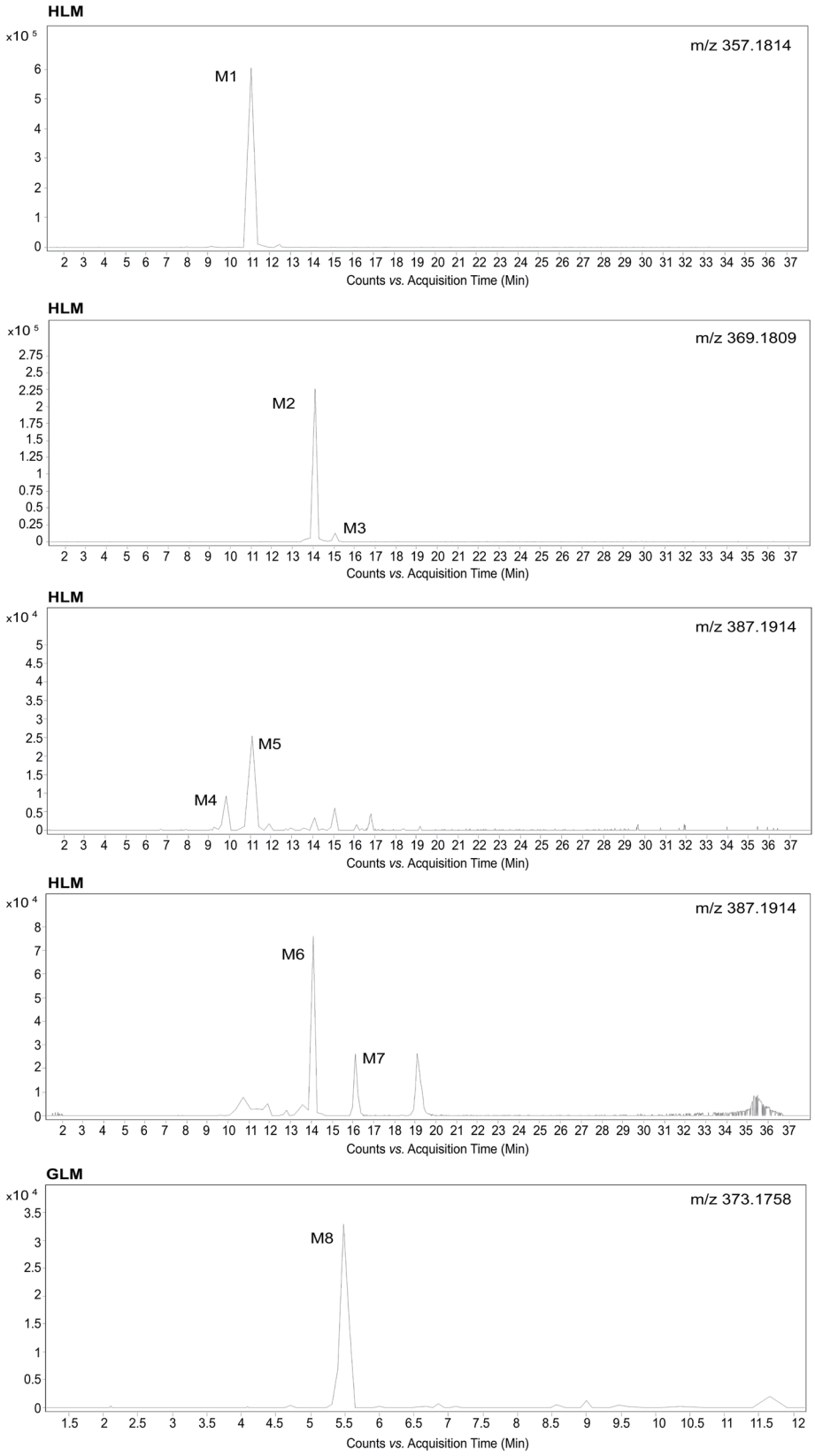

Fig. 1. The extracted ion chromatograms of M1-M7 (HLM) and M8 (GLM). GLM, goat liver microsomes; HLM, human liver microsomes.

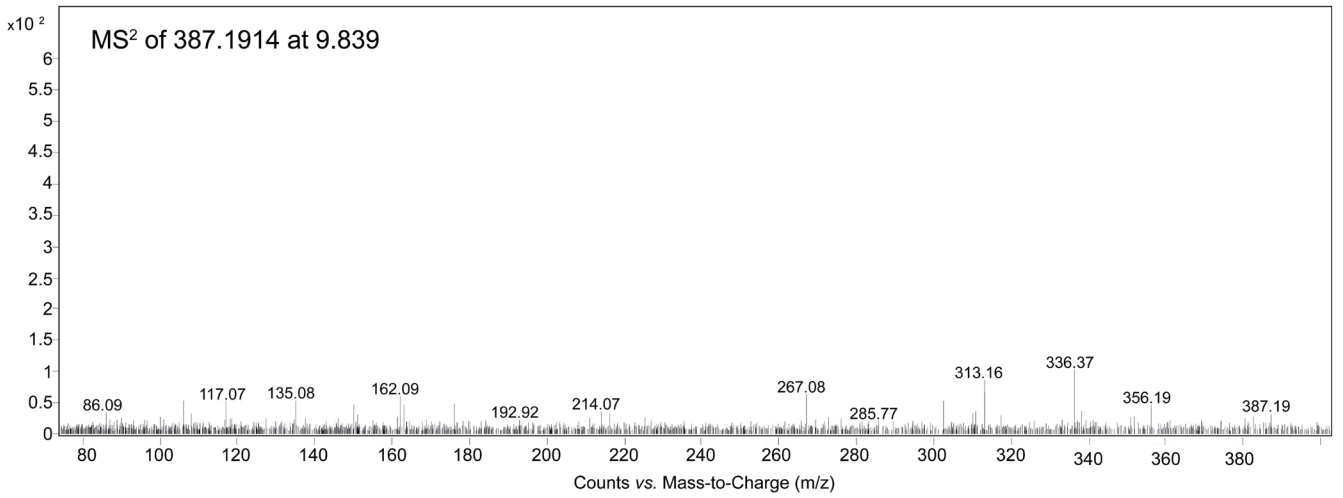

Fig. 2. The product ion spectra of metabolite M4.

increased by approximately fivefold, and the M1 peak intensity in GLM increased by twofold. Both PLM and GLM contained similar amounts of M2, while the content detected in HLM was about fivefold higher than in PLM and GLM. HLM had the highest amount of M3, followed by GLM and PLM. Similar levels of M5 were detected in PLM and GLM, while approximately tenfold higher levels of M5 were detected in HLM compared to PLM and GLM. The content of M6 was identified in the following order: human > pig > goat, with the content detected in HLM approximately eightfold higher than in PLM, and approximately tenfold higher than in GLM. M4 was detected only in HLM and GLM, with the content in HLM approximately eightfold higher than in GLM. M7 was detected only in HLM. M8 was detected in GLM and PLM, with the content of M8 in GLM approximately fivefold higher than in PLM. The specific relative peak intensities of M1–M8 are shown in Table 2.[14]

Discussion

Seven metabolites were found in rat liver microsomes, seven in human liver microsomes,[14] seven in goat liver microsomes, and six in pig liver microsomes, suggesting that there is no significant difference in the metabolism of humantenirine among different species. As shown in Figure 3, the main metabolic pathways of humantenirine in liver microsomes of humans, pigs, and goats were demethylation, oxidation, and dehydrogenation. Current metabolic studies

Table 1. The retention time (RT), elemental compositions, observed masses, predicted masses, mass errors of humantenirine and its metabolites in HLM, PLM, GLM

Compound	RT (m)	Elemental composition	Observed mass	Predicated mass	Mass error (ppm)	Product ion
Humantenirine	16.1	$C_{21}H_{26}N_2O_4$	371.1954	371.1965	3.06	340.1776, 325.1535, 311.1410, 176.0699, 164.1061, 108.0801
M1	11.0	$C_{20}H_{24}N_2O_4$	357.1814	357.1809	−1.45	326.1608, 311.1376, 297.1284, 178.1207, 164.1063, 162.0544, 108.0808
M2	14.1	$C_{21}H_{24}N_2O_4$	369.1802	369.1809	1.86	338.1627, 323.1363, 309.1601, 176.1065, 164.1068, 162.0936, 108.0809, 106.0669
M3	15.0	$C_{21}H_{24}N_2O_4$	369.1810	369.1809	−0.32	338.1599, 323.1443, 311.1803, 309.1572, 293.1284, 108.0642
M4	9.8	$C_{21}H_{26}N_2O_5$	387.1919	387.1914	−1.17	341.1487, 188.0668, 180.0922, 176.0718, 162.0884
M5	11.0	$C_{21}H_{26}N_2O_5$	387.1920	387.1914	−1.43	356.1674, 341.1505, 338.1610, 325.1517, 310.1772, 180.1011, 176.0714, 162.0851, 106.0701
M6	14.1	$C_{21}H_{24}N_2O_5$	385.1768	385.1758	−2.61	354.1412, 339.1323, 325.1490, 323.1596, 176.0663, 120.0745, 106.0642
M7	16.1	$C_{21}H_{24}N_2O_5$	385.1751	385.1758	1.82	354.1554, 339.0008, 336.1749, 311.1277, 178.0848, 174.0796, 136.0764, 122.0629, 106.0713
M8	5.5	$C_{20}H_{24}N_2O_5$	373.1762	373.1758	−1.08	342.1651, 327.1279, 311.1377, 282.1454, 194.1093, 180.0991

GLM, goat liver microsomes; HLM, human liver microsomes; PLM, pig liver microsomes.

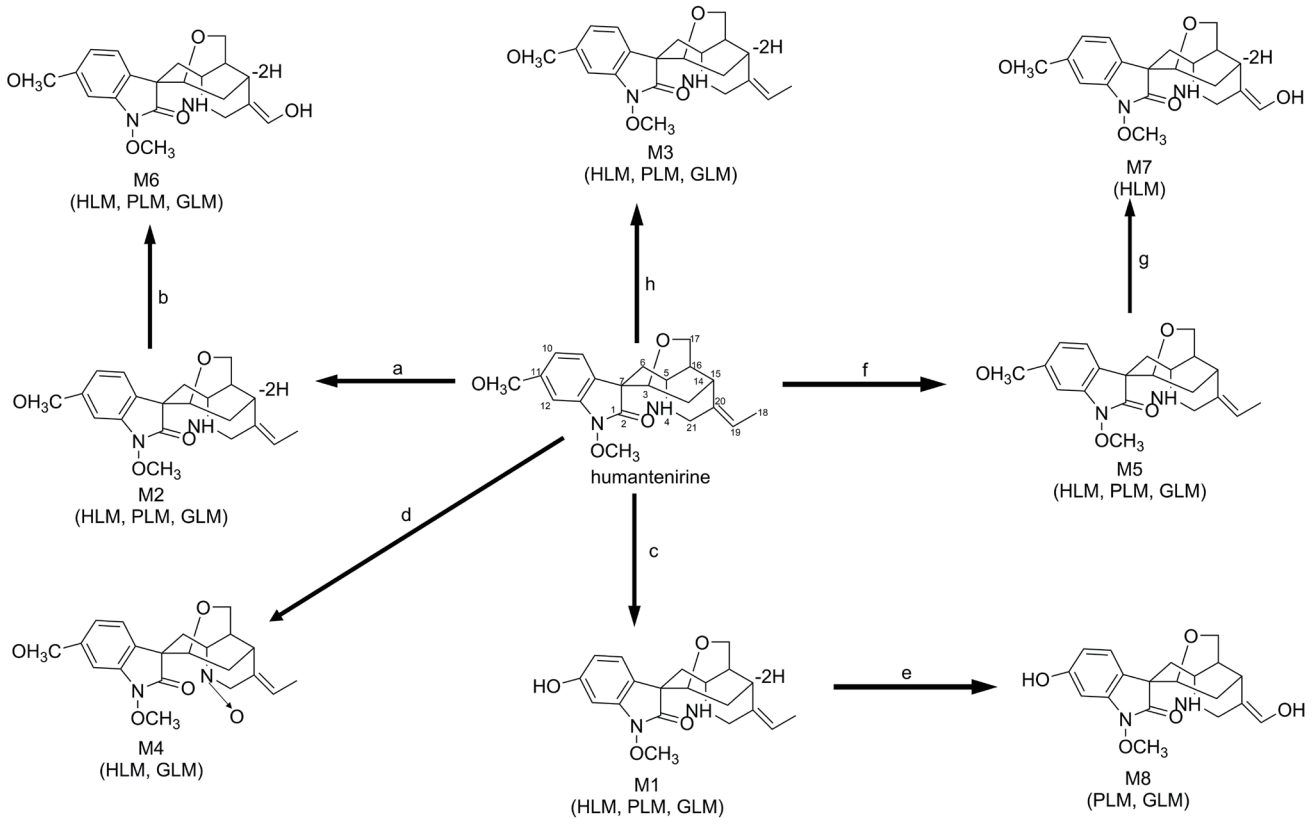

Fig. 3. The possible metabolic pathways of humantenirine in HLM, PLM, GLM. (a, h, g) dehydrogenation; (b, d, e, f) oxidation; (c) demethylation. GLM, goat liver microsomes; HLM, human liver microsomes; PLM, pig liver microsomes.

on *G. elegans* alkaloids, including koumine, gelsemine, and gelsenicine, have found that their major metabolic pathways include oxidation, demethylation, dehydrogenation, N-demethylation, and hydrogenation.[16,20] Metabolic studies on the multi-component nature of *G. elegans* also showed that the major metabolic pathways of *G. elegans* in goats are oxidation, glucuronidation, demethylation, dehydrogenation, and hydrogenation.[21] It is inferred that the metabolic pathways of *G. elegans* alkaloids are similar across species and that demethylation, dehydrogenation, and oxidation are common metabolic pathways for *G. elegans* alkaloids.

Table 2. The relative peak intensity of humantenirine and its metabolites in HLM, PLM, GLM, and rat live

Compound	HLM_S	PLM_S	GLM_S	Rat live[14]
Humantenirine	3.25×10^6	1.3×10^6	2.0×10^6	1.0×10^6
M1	6.0×10^5	3.3×10^6	1.8×10^6	8.0×10^5
M2	3.1×10^5	2.5×10^4	2.9×10^4	1.0×10^5
M3	0.2×10^5	1.3×10^4	1.6×10^4	0.6×10^4
M4	1.7×10^4	ND	0.2×10^4	–
M5	5.2×10^4	6.3×10^3	0.6×10^4	7.0×10^4
M6	8.0×10^4	1.0×10^4	8.5×10^3	2.1×10^4
M7	2.8×10^4	ND	ND	3.0×10^4
M8	ND	6.8×10^3	3.3×10^4	1.0×10^5

GLM, goat liver microsomes; HLM, human liver microsomes; ND, not detected; PLM, pig liver microsomes.

Demethylation was the major metabolic pathway in liver microsomes of the three species. According to previous studies, demethylation may be the detoxification pathway for gelsenicine.[22] In the present study, the demethylated metabolite M1 of humantenirine was found to have a higher peak intensity in the liver microsomes of goats and pigs than in humans, suggesting that demethylation is the detoxification pathway for *G. elegans* alkaloids. Additionally, it has been reported that the dehydrogenation metabolic pathway may be a toxic pathway for rankinidine.[13] The peak intensities of the dehydrogenated metabolites, M2 and M3, were higher in rat and human liver microsomes than in pig and goat liver microsomes, which suggests that this may be the main toxicity pathway for *G. elegans* alkaloids. The relatively high peak intensities of dehydro-metabolites and relatively low peak intensities of demethylated metabolites in rat and human liver microsomes suggest that humans may be more susceptible to toxicity than pigs and goats. This difference may be related to variations in the composition and levels of the metabolic enzyme CYP450 in the livers of different species.[23] However, this is only a conjecture, and further studies are needed to confirm these results. The predicted ADMET (absorption, distribution, metabolism, excretion and toxicity) properties of *G. elegans* revealed that all *G. elegans* alkaloids were predicted to be substrates of CYP3A4, which is the major metabolizing enzyme of *G. elegans* alkaloids, and that the catalytic activity of CYP3A4 shows significant species differences.[24] Additionally, it was found that koumine and humantenmine could be

detoxified by CYP3A4/5-mediated metabolic reactions,[25,26] and CYP3A4-mediated metabolic reactions could also reduce the toxicity of gelsemine.[17] Therefore, we hypothesize that the degree of metabolism of *G. elegans* in different species is also related to the level of CYP3A4/5.

Conclusions

This study used HPLC/QqTOF-MS for the first time to study the *in vitro* metabolism of humantenirine in different species. In HLM, GLM, and PLM, eight metabolites were identified, and three metabolic pathways (demethylation, dehydrogenation, and oxidation) were discovered. There were no significant differences in metabolic pathways between the species. This study provides a substantial theoretical basis for understanding the toxicological differences of *G. elegans* across different species.

Acknowledgments

None.

Funding

This work was supported by the National Natural Science Foundation of China (Grant No. 31972737).

Conflict of interest

ZYL has been an editorial board member of *Future Integrative Medicine* since February 2023. The author declares no other conflicts of interest.

Author contributions

Experiments conceiving and designing (ZYL), experiments performance (XJQ), manuscript drafting (YFW), and critically editing (MTZ, ZYL).

Data sharing statement

No additional data are available.

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