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RESEARCH ARTICLE

Characterization of *in vitro* and *in vivo* metabolism of leelamine using liquid chromatography-tandem mass spectrometry

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ABSTRACT

1. Leelamine is a diterpene compound found in the bark of pine trees and has garnered considerable interest owing to its potent anticancer properties. The aim of the present study was to investigate the metabolic profile of leelamine in human liver microsomes (HLMs) and mice using liquid chromatography-tandem mass spectrometry (LC-MS/MS).
2. We found that leelamine undergoes only Phase I metabolism, which generates one metabolite that is mono-hydroxylated at the C9 carbon of the octahydrophenanthrene ring (M1) both *in vitro* and *in vivo*. The structure and metabolic pathway of M1 were determined from the MS^n fragmentation obtained by collision-induced dissociation using LC-MS/MS in HLMs.
3. Cytochrome p450 (CYP) 2D6 was found to be the dominant CYP enzyme involved in the biotransformation of leelamine to its hydroxylated metabolite, whereas CYP2C19, CYP1A1, and CYP3A4 contributed to some extent.
4. Moreover, we identified only one metabolite M1, in the urine, but none in the feces. In conclusion, leelamine was metabolized to a mono-hydroxyl metabolite by CYP2D6 and mainly excreted in the urine.

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KEYWORDS
Leelamine; cytochrome P450; human liver microsomes; metabolite; LC-MS/MS

Introduction

Drug metabolism and pharmacokinetics (DMPK) have a major role in the initial stages of drug discovery and development (Prasad et al., 2011). DMPK studies are performed using *in vitro*, *in silico,* or *in vivo* models, and involve metabolic stability determination, enzyme phenotyping, investigation of drug–drug interactions, metabolite profiling, and plasma protein-binding assays. Metabolic profiling of xenobiotics is a prominent tool for the toxicological evaluation of drugs that focuses on their metabolic pathways and determines systemic metabolites (Lin and Lu, 1997; Prasad et al., 2011; Tiller and Romanyshyn, 2002). In addition, based on *in vitro* studies, suitable animal models are subsequently selected for further *in vivo* studies that reliably predict the metabolic pathways in humans (Lee et al., 2011). The human liver microsomes (HLMs) are a liver fraction and the most widely used *in vitro* system for metabolic profiling studies (Asha and Vidyavathi, 2010; Lee & Liu, 2007). This system is enriched with metabolic enzymes, such as cytochrome P450 (CYP) isoforms, flavin mono-oxygenase, and UDP-glucuronosyltransferase (UGT), which are mainly responsible for the metabolism of xenobiotics and endogenous substances.

Leelamine (Figure 1), a dehydroabietylamine derivative of dehydroabietic acid, is found in the resins of pine trees (Kovaleva et al., 2017). It is a pyruvate dehydrogenase kinase 4 (PDK4) inhibitor and has been found to exhibit hypoglycemic activity in ob/ob mice and inhibit glyceroneogenesis in isolated adipocytes by activating the pyruvate dehydrogenase complex (PDC) (Cadoudal et al., 2008; Jeoung & Harris, 2010). Leelamine as a drug has been of considerable interest recently because it has been effective in the treatment of melanoma by disrupting cholesterol homeostasis in cancer cells (Gowda et al., 2017; Kuzu et al., 2014). It exhibits a strong anticancer effect on human breast cancer cell lines by generating reactive oxygen species and inducing B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax)/Bcl-2 homologous antagonist/killer (Bak)-dependent apoptosis (Sehrawat et al., 2017). Moreover, our previous study in mice showed that leelamine is a potent and selective inducer of CYP2B activity, but has a low oral bioavailability of 7.6% (Sim et al., 2015; Song et al., 2013).

Although numerous studies have investigated the biological activity of leelamine, to the best of our knowledge, no studies have focused on its metabolic pathway (Gowda

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Supplemental data for this article can be accessed here.

et al., 2014a; Kuzu et al., 2014; Sehrawat et al., 2017). Therefore, in this study, we identified the structures of the metabolites of leelamine in HLMs using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and investigated the CYP enzyme isoforms involved in its biotransformation using purified human recombinant cDNA-expressed CYPs. Furthermore, we investigated the *in vivo* metabolic profile of leelamine using urine and feces analysis after intraperitoneal (*i.p.*) treatment (10 mg/kg) of mice. This study provides a general overview of the *in vitro* and *in vivo* metabolic fate of leelamine.

Materials and methods

Materials

Leelamine hydrochloride was repurified from a commercially available reagent (Song et al., 2013). The NADPH (β-reduced nicotinamide adenine dinucleotide phosphate) regeneration system (NGS) was purchased from Promega Corp. (Madison, WI). Reserpine was obtained from Sigma-Aldrich (St. Louis, MO). Pooled HLMs (BD Ultra Pool™ HLM 150®) and purified human recombinant cDNA-expressed CYP1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 were purchased from Corning Gentest (Woburn, MA). High-performance-LC (HPLC)-grade water and acetonitrile (ACN) were acquired from Fischer Scientific (Pittsburgh, PA).

Animals

Specific pathogen-free, 5-week-old, male ICR mice (28–33 g) were purchased from the Orient Co. (Seoul, Korea) and acclimated for 1 week in a temperature- and humidity-controlled environment (23 ± 3 °C and 50 ± 10% relative humidity (RH) under a 12-h light/dark cycle at an intensity of 150–300 Lux. All animal handling and care protocols conformed with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health (NIH), and were approved by the Animal Care and Use Committee of Kyungpook National University (NO 2016-0089).

In vitro *metabolism of leelamine*

Leelamine (final concentration, 50 μM) was incubated with 1 mg/mL HLMs in addition to NGS solution consisting of glucose-6-phosphate dehydrogenase (1 U/mL), glucose 6-phosphate (0.1 M), and β-NADPH (10 mg/mL). The reaction mixture (200 μL) was incubated at 37 °C for 60 min, and all experiments were carried out in triplicate. The reaction was terminated by adding 400 μL 100% ACN, and then the mixture was vortexed and centrifuged for 10 min at 13,000 rpm. A 550 μL sample of the supernatant was transferred to a new e-tube and was dried using a speed-vacuum concentrator. The dried sample was dissolved in 100 μL of 50% ACN (0.1% formic acid). The supernatant was vortexed and centrifuged (13,000 rpm, 10 min, 4 °C), and then, a 10 μL sample was injected into a C18 column for the LC-MS/MS analysis.

Metabolism of leelamine in human recombinant cDNA-expressed CYP

To determine the possible CYP isoforms involved in the metabolism of leelamine, we incubated 10 μM of leelamine with 10 pmol each of 10 purified recombinant human hepatic CYP isoforms (CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 CYP3A4, and CYP3A5) in the presence of NGS for 60 min at 37 °C in a reaction volume of 200 μL. The reaction was stopped by adding 400 μl of 100% ice-cold ACN containing 0.1% formic acid. Following centrifugation at 13,000 rpm for 10 min, 550 μL of the supernatant was evaporated using a speed-vacuum concentrator. The dried samples were reconstituted with 50% ice-cold ACN containing 0.1% formic acid and analyzed using LC-MS/MS to detect the metabolites.

In vivo *metabolism of leelamine*

After examining the *in vitro* metabolism of leelamine in HLM, we selected mice as the *in vivo* model to study the metabolic profiling of leelamine in urine and feces. The ICR mice were randomly divided into control and test groups of three mice each. Leelamine (10 mg/kg) was prepared in saline and administered *i.p.* to the test group, whereas saline was administered to the control group. After *i.p.* administration, the feces and urine were collected over time intervals of 0–12, 12–24, and 24–48 h. The urine and feces were subjected to solid-phase extraction, followed by drying using a speed-vacuum concentrator, and then the samples were reconstituted with 10% methanol (MeOH) containing 0.1% formic acid. The samples were finally analyzed using LC-MS/MS to identify *in vivo* metabolites and determine the excretion route.

Instruments

A Thermo LTQ XL™ (Thermo Fisher Scientific Inc., Waltham, MA) linear ion trap was used to detect and elucidate the structures of leelamine and its metabolites. It was operated in the positive-ion electrospray mode, and data-dependent MS^2 and MS^3 collision-induced dissociation (CID) was programed to achieve the elucidation of metabolite structure. The mobile phase consisted of water containing 0.1% formic acid (mobile phase A) and 100% ACN with 0.1% formic acid (mobile phase B) with gradient elution at a flow rate of 0.22 mL/min. The gradient condition was as follows: 5% of B at 0–0.75 min, 5–95% of B at 0.75–15 min, 95% of B at 15–16 min, 95–5% of B at 16–16.5 min, and 5% B at 16.50–22.0 min. The analytes were separated using a Kinetex® C18 column (150 mm × 2.1 mm, 2.6-μm, Phenomenex, Torrance, CA) maintained at 40 °C. Nitrogen was used as the sheath, auxiliary, and sweep gas at flow rates of 60, 20, and 5 (arbitrary units), respectively. The mass spectrometer was operated in the full scan mode at 60–700 *m/z*. Ion spray voltage was adjusted to 5 kV, and the capillary temperature was 300 °C. The data were acquired and analyzed using the Xcalibur 3.0.63 (Thermo Fischer Scientific, San Jose, CA).

Results

Identification of hydroxylated leelamine in HLMs

In the first step, the Phase I metabolic stability of leelamine (5 μM) was determined using 0.25 mg/mL HLMs in the presence of NGS. The metabolic stability was calculated from the peak area ratio of leelamine to reserpine as an internal standard. In a time span of 60 min, leelamine was decreased by more than 25%, suggesting that leelamine underwent Phase I metabolism (Supplementary Figure S1). Following incubation of leelamine with HLMs in the presence of NGS for 60 min, leelamine was metabolized to only one monohydroxylated form, M1, and its protonated ion was observed at *m/z* 302.6 with a retention time of 9.4 min (Figure 2). No M1 peak was observed in samples without NGS, while we observed time-dependent formation of M1 in HLM in the presence of NGS for 60 min (Supplementary Figure S2A). Additional evidence to support the formation of M1 by CYPs was obtained by incubating the sample with SKF-525A, a nonspecific CYP inhibitor, which significantly decreased the formation of M1 (Figure 3(A)). No phase II conjugates of leelamine, especially glucuronyl-conjugates, were detected in HLMs incubated with its cofactor, UDPGA, which accorded well to the Phase II metabolic stability of leelamine (Supplementary Figure S1B).

After confirming the generation of M1 by Phase I metabolism in HLMs, we identified the possible CYP enzyme isoforms involved in the metabolism of leelamine (Figure 3(B)). Following the incubation of 10 μM leelamine with 10 pmol of specific human recombinant CYP isoforms in the presence of NGS, M1 was formed in CYP1A1, CYP2C19, CYP2D6, and CYP3A4 enzyme samples. CYP2D6 was found to be the predominant enzyme to metabolize leelamine to M1. CYP2C19 partly participated in the metabolism of leelamine to M1, whereas CYP1A1 and CYP3A4 contributing to a small extent. When we re-incubated 5 μM leelamine in each CYP isoforms at 37 °C for 60 min to confirm the contribution of CYP on leelamine metabolism, the metabolic stability of leelamine showed strong decrease in CYP2D6 (Supplementary Figure S3), which correctly corresponded to time-dependent increase of M1 in CYP2D6 (Supplementary Figure S2B).

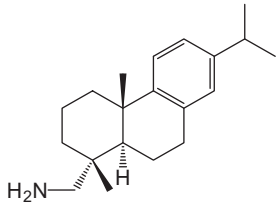

Figure 1. Chemical structure of leelamine.

Elucidation of M1 structure

Prior to elucidating the MS/MS fragmentation, CID values of leelamine in the positive ion mode were optimized to 15 and 30 for MS^2 dissociation of *m/z* 286.2 → 173.1 and MS^3 dissociation of *m/z* 286.2 → 173.1 → 130.9 by infusion of 5 μM leelamine in 50% ACN with 0.1% formic acid, respectively. Leelamine was eluted at 11.9 min with a protonated molecule $[M+H]^+$ at *m/z* 286 as shown in the extracted ion chromatograph (EIC, Figure 2). The CID-induced MS/MS fragmentation spectra generated ions at *m/z* 269.2 with a loss of NH_3

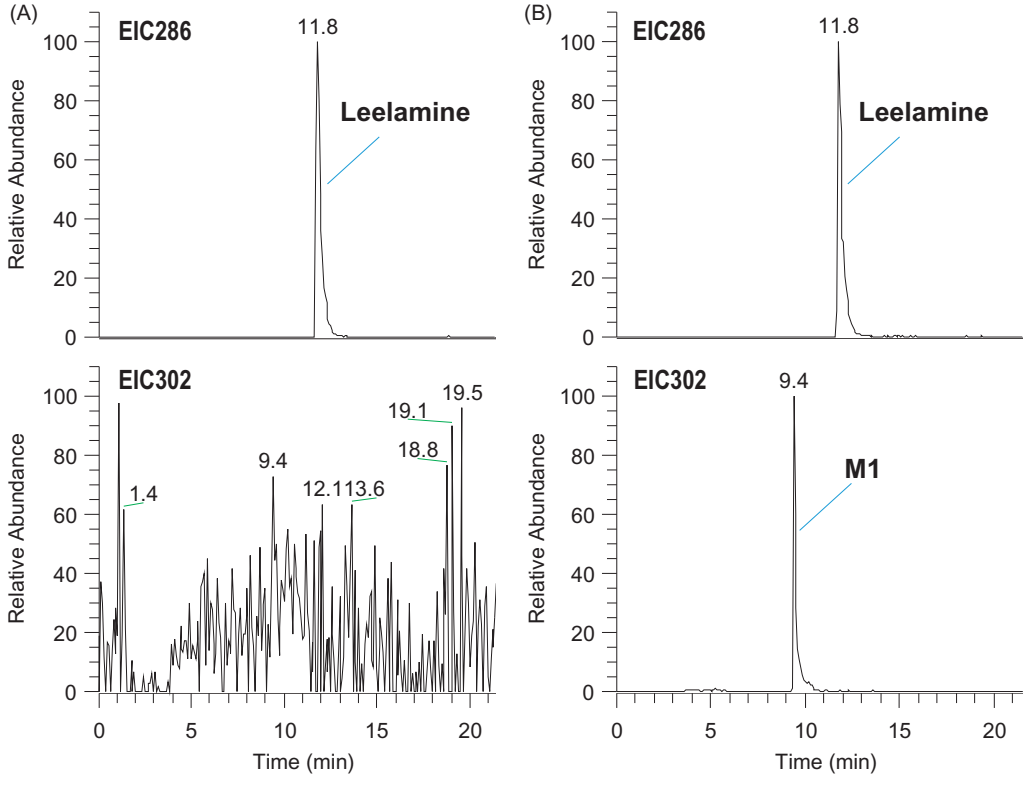

Figure 2. Extracted ion chromatograms of leelamine (50 μM) and its hydroxyl metabolite (M1) after 60-min incubation with 1 mg/mL human liver microsomes (HLMs) (A) without and (B) with β-reduced nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system (NGS).

(−17 Da), at *m/z* 187.1 with a loss of $C_6H_{14}N$ (−100 Da), and a prominent ion at *m/z* 173.1 with a loss of $-C_7H_{15}N$ (−113 Da) ions. Therefore, the ion at *m/z* 173.1 was chosen for an additional MS^3 analysis, which generated the peak with the highest intensity at *m/z* 131.0 by the elimination of C_3H_8 (−44 Da). The postulated fragmentation pathway of leelamine in the positive ion mode is summarized in Figure 4(A).

We detected only one mono-hydroxyl metabolite of leelamine in the LC-MS/MS analysis in this study. M1 was formed by the addition of one oxygen ion (+16 Da) with an *m/z* of 302.6 and a retention time of 9.4 min (Figure 2). The postulated fragmentation pathway of M1 in the positive ion mode is summarized in Figure 4(B). The fragmentation pattern of M1 was similar to that of leelamine, resulting in ions *m/z* 285.3, 202.9, and 189.2 by the addition of 16 Da at *m/z* 269.2, 187.1, and 173.1 fragments of leelamine, respectively, and *m/z* 202.9 and 189.2 indicated the mono-hydroxylation of the tetrahydronaphthalene moiety. The ions detected at *m/z* 131 of M1 with the loss of C_3H_8O (−58 Da) from *m/z* 189.2 using the MS^3 analysis were the same as those in leelamine, indicating no hydroxylation of the cumene moiety. This pattern of fragmentation finally supported the monohydroxylation of the octahydrophenanthrene ring at the C9 carbon (Figure 3(B)).

In vivo *metabolism of leelamine*

To investigate the *in vivo* metabolites and excretion pathway, urine and feces analyses were carried out following *i.p* administration of leelamine (10 mg/kg) in mice. Although we monitored the masses of the major potential metabolites, we only identified mono-hydroxyl leelamine (M1) in the urine (Figure 5), which was similar to the finding of our *in vitro* study. In addition, we found that leelamine was minimally eliminated through feces within 12 h of administration (Supplementary Figure S4). However, leelamine and M1 showed high urinary excretion within 12 h, with almost complete excretion within 48 h, and M1 is excreted approximately 6 folds higher than leelamine in urine (Figure 5). Interestingly, although leelamine was excreted via both urine and feces, we only detected M1 in the urine samples and not in feces, which suggests that the main excretion pathway of M1 is urinary. The *in vitro* and *in vivo* metabolic pathways of leelamine are summarized in Figure 6.

Discussion

Leelamine has recently attracted widespread interest owing to its potent anticancer properties, and has been successfully formulated as a nanoparticle-based liposomal drug delivery system (Gowda et al., 2014b). Most studies on leelamine have been limited to the elucidation of its anticancer mechanism (Gowda et al., 2014a; Sehrawat et al., 2017), but its metabolic pathway has not been established in both *in vitro* and *in vivo* models. Therefore, it would be useful to understand the metabolic fate of leelamine in HLMs and investigate its metabolic elimination profile *in vivo*.

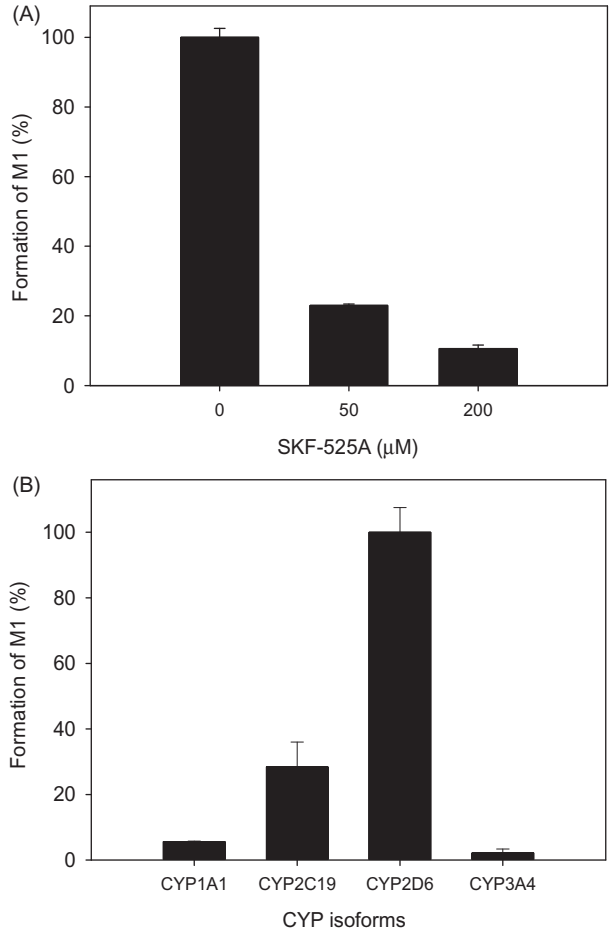

Figure 3. Effect of SKF-525A (50 and 200 μM), a non-specific cytochrome P450 (CYP) inhibitor, on metabolism of leelamine in HLMs (A). Relative formation of M1 by CYP isoforms involved in the metabolism of leelamine to its hydroxylated form (B). Results are represented as mean ± S.E.M. of triplicate analysis.

In this study, we identified that leelamine was metabolized to only one mono-hydroxylated metabolite, M1, generated by the addition of one oxygen ion (16 Da) at the C9 carbon of the octahydrophenanthrene ring in the HLMs (Figure 6). From our study, the MS^3 spectra of product ions of leelamine and the key ions at *m/z* 267, 189, 131, and 189 strongly support the M1 structure. However, further extensive works should be carried out to precisely confirm the structure of M1, by analyzing NMR spectroscopy of isolated metabolite or comparing the chromatograph of a chemically synthesized standard.

From the *in vivo* metabolic study, we found that leelamine was mainly excreted in the urine and only one metabolite, M1, was identified in the mouse urine. Thus, the high rate of metabolism of leelamine to its hydroxylated metabolite M1 by CYP2D6 in the liver could account for its low bioavailability (7.6%), as demonstrated in our previous study [9]. Hydroxylation of leelamine increases the hydrophilicity of M1, resulting in its higher urinary elimination. The urinary concentration of M1 was higher than that of leelamine, which could be attributable to the high rate of metabolism of leelamine to M1 (Figure 5).

Additionally, the *in vivo* studies of the urine showed that leelamine and its hydroxylated metabolite were mainly

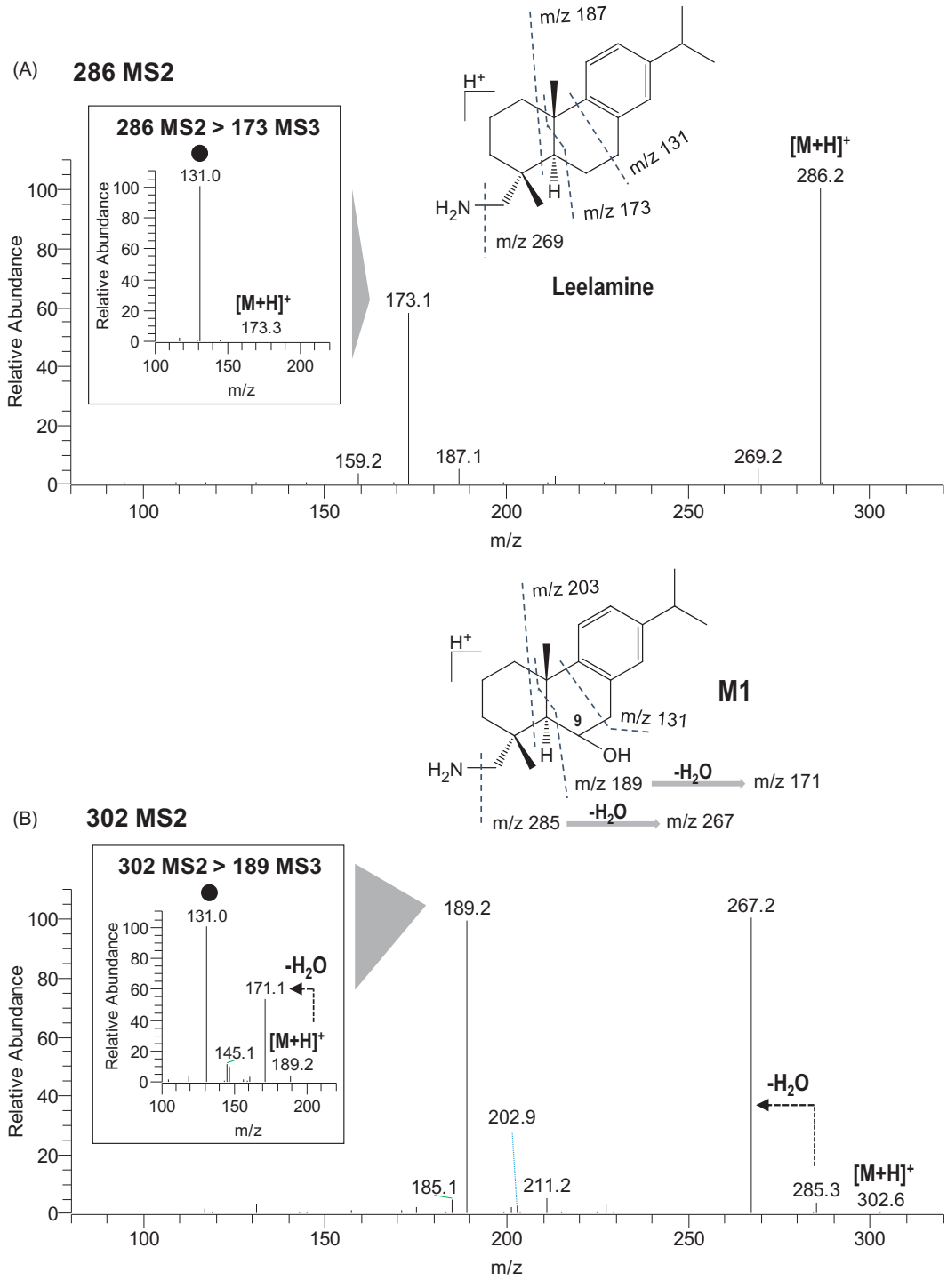

Figure 4. Tandem mass spectrometry (MS/MS) and MS^n spectra of (A) protonated leelamine and (B) M1. Proposed mechanism using hybrid-linear ion trap mass spectrometer.

eliminated through the urine within 48 h of *i.p.* administration, which is an important factor to be considered in establishing the therapeutic dose of leelamine. Further studies should be carried out to investigate the pharmacological activity, potency, toxicity, and PK of the hydroxyl metabolite, M1, of leelamine.

The CYP2D6 enzyme metabolizes more than 20% of clinically used drugs and exhibits high polymorphism that is used to categorize the population, according to the degree of activity of the enzyme, as poor, intermediate, extensive, and ultrarapid metabolizers (PM, IM, EM, and UM, respectively) (Bernard et al., 2006). The genetic polymorphism of CYP2D6 differs widely among different ethnicities and has varying effects on drug metabolism by the enzyme. Adverse drug effects or therapeutic loss could develop because of CYP2D6 polymorphisms (Ingelman-Sundberg, 2005; Zanger & Schwab, 2013). One notable example of the effect of CYP2D6 polymorphism on drug metabolism is that on tamoxifen. CYP2D6 metabolizes tamoxifen, a leading chemotherapeutic drug for breast cancer, to its active form endoxifen. However, patients

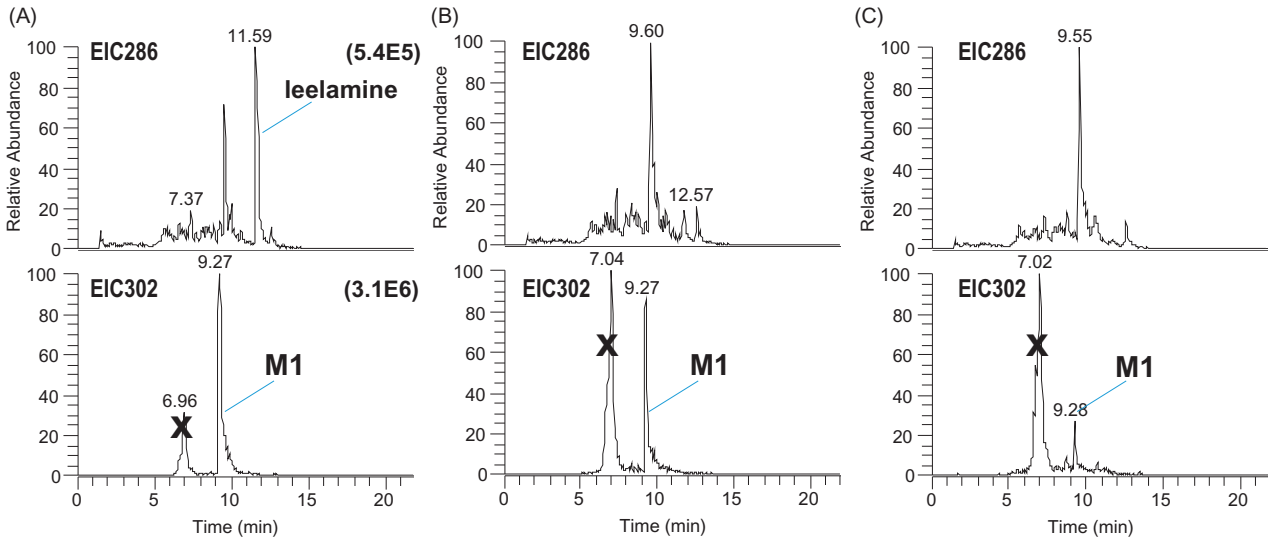

Figure 5. Extracted ion chromatograph (EIC) of leelamine and M1 from urine samples of leelamine-treated mice at (A) 0–12, (B) 12–24, and (C) 24–48 h.

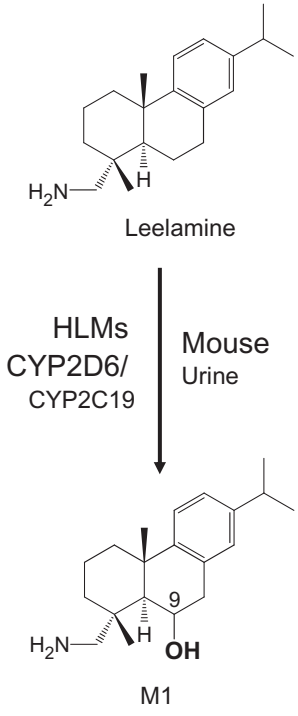

Figure 6. Postulated metabolic pathways of leelamine in human liver microsomes (HLMs).

with a PM status fail to respond to the drug, causing a high relapse rate of the disease (Beverage et al., 2007). Similarly, in this study, we found that leelamine is highly metabolized to its hydroxylated form by a CYP2D6 isoform. It is likely that the metabolic profile of leelamine in humans could exhibit interindividual variation. Therefore, acute research studies should be performed to determine the leelamine dose that shows effective therapeutic activity.

Conclusion

In summary, the present *in vitro* study revealed CYP2D6 to be the main CYP isoform involved in the metabolism of leelamine by mono-hydroxylation of the octahydrophenanthrene ring at the C9 carbon. Furthermore, the metabolic pathway of leelamine in mice was studied using urine and feces analysis, which revealed urinary excretion as the major elimination route of the metabolite compared to fecal excretion. Hence, the metabolism of leelamine was found to generate only the mono-hydroxyl metabolite in both *in vitro* and *in vivo* studies.

Disclosure statement

No potential conflict of interest was reported by the authors.

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