Vol. 9, No. 3, 2018
ISSN 2233-4203/ e-ISSN 2093-8950
LETTER www.msletters.org | Mass Spectrometry Letters

In vitro Metabolism of Methallylescaline in Human Hepatocytes Using Liquid Chromatography-High Resolution Mass Spectrometry

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Received September 7, 2018; Revised September 28, 2018; Accepted September 28, 2018
First published on the web September 30, 2018; DOI: 10.5478/MSL.2018.9.3.86

Abstract : Methallylescaline, 2-(3,5-dimethoxy-4-[(2-methylprop-2-en-1-yl)oxy]phenyl)ethanamine, is a new psychoactive substance with potent agonist of 5-HT receptor, but there is little information on its pharmacological effect, metabolism, and toxicity. It is necessary to characterize the metabolic profiling of methallylescaline in human hepatocytes using liquid chromatography-high resolution mass spectrometry. Methallylescaline was metabolized to three hydroxy-methallylescaline (M1-M3) and dihydroxy-methallylescaline (M4) via hydroxylation in human hepatocytes. CYP2D6, CYP2J2, CYP1A2, and CYP3A4 enzymes were responsible for the metabolism of methallylescaline. The metabolites as well as methallylescaline would be used for monitoring the abuse of methallylescaline,

Keywords : methallylescaline, *in vitro* metabolism, liquid chromatography-high resolution mass spectrometry

Introduction

New psychoactive substances (NPS) are abused substances that have similar effects to controlled drugs such as cocaine, cannabis, and amphetamine-type stimulants. According to United Nations Office on Drugs and Crime (UNODC) report, the numbers of NPS was doubled-up from 260 different NPS in 2012 to 479 different substances in 2017.[1]

NPS can be categorized by chemical structure: synthetic cannabinoids, synthetic cathinones, tryptamines, and phenethylamines, etc. Still synthetic cannabinoids are the largest group in NPS, phenethylamines become more important recently and account for 28.4% of NPS reported to UNODC by the end of 2017.[1]

Phenethylamines are the typical agonist for 5-HT_{2A} receptor, resulting in psychoactive effects, and their structure-activity relationship is well-characterized.[2,3] After the finding that *N*-benzyl substitution of phenethylamine significantly improved not only binding affinity but also functional activity of 5-HT receptor, several derivatives having methoxy, hydroxy, and fluorine moiety have been synthesized and evaluated structure-activity relationship.[3-5]

Methallylescaline, 2-(3,5-dimethoxy-4-[(2-methylprop-2-en-1-yl)oxy]phenyl)ethanamine, is a methylallyl derivative of mescaline, which is a representative 5-HT_{2A} agonist. There is little information on the pharmacological effect, metabolism, and toxicity of methallylescaline. However, owing to its psychedelic effect, its use is gradually controlled since 2013.[6]

It is critical to develop a bioanalytical method for sensitive and selective determination of illegal substances in the control of illicit drugs. It is essential to understand metabolic characteristics of illegal substances to develop their bioanalytical method, because lots of illegal substances are extensively metabolized in the body.[7-11] The purpose of this study was to characterize the metabolic pathways of methallylescaline in human hepatocytes using liquid chromatography-high resolution mass spectrometry (LC-HRMS).

Experimental

Materials

Methallylescaline was obtained from Maidan Chemicals Co., Ltd. (Ukraine). LiverPool™ 50-donor pooled

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cryopreserved human hepatocytes (lot no. HQE), InVitroGRO™ HT Medium, and InVitroGRO™ KHB were obtained from Bioreclamation IVT (Brussels, Belgium). Honokiol (positive control for metabolic stability assay) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, methanol, and water (LC-MS grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Other chemicals used were of the highest grade available.

Metabolic stability in human hepatocytes

Human hepatocytes were thawed carefully using InVitroGRO™ HT Medium according to the manufacturer's instructions. Thawed human hepatocytes were suspended in Krebs-Henseleit buffer to a final density of 1.0×10^6 cells/mL. Subsequently, 60 μL of hepatocyte suspension and 60 μL methallylescaline (4 μM) in Krebs-Henseleit buffer were added to 96-well plates and incubated in triplicate for 0, 5, 15, 30, 60, 120, and 180 min in a humidified CO_2 static incubator at 37°C. Honokiol (final concentration: 2 μM) was separately incubated for 0, 15, 30, 60, and 120 min as a positive control.[12] Incubations were stopped by adding 120 μL ice-cold acetonitrile to each well, followed by 5-min sonication and centrifugation at 15,000 × g for 10 min at 4°C. Then, 100 μL of supernatant was mixed with 100 μL of distilled water. Aliquots (2 μL) of each sample were then injected into LC-MS/MS system. The peak areas ratio of each substance to IS at each sampling point were measured and applied to calculate following parameters. The elimination half-life ($t_{1/2}$), intrinsic clearance (Cl_{int}), hepatic clearance (CL_{hep}), and hepatic extraction ratio (ER) of methallylescaline were calculated using well-stirred model by following equations:

$$Cl_{(\mathrm{int})}(mL/min/kg) = \frac{ln2}{t_{1/2}} \times \frac{mL\ incubation}{hepatocyte(10^6 cells)} \frac{139 \times 10^6 cells}{g\ liver} \times \frac{25.7 g\ liver}{kg\ body\ weight}$$

$$Cl_{\mathrm{hep}} = \frac{Q_h \times Cl_{\mathrm{int}}}{Q_h + Cl_{\mathrm{int}}},\ Q_h = 20.7\ mL/min/kg$$

$$ER = \frac{Cl_{\mathrm{hep}}}{Q_h} \times 100$$

Q_h refers to hepatic blood flow.[13]

Metabolite identification in human hepatocytes

Human hepatocytes were incubated as described in metabolic stability experiment. 60 μL of human hepatocytes suspension (1.0×10^6 cells/mL) and 60 μL of 20 μM methallylescaline in Krebs-Henseleit buffer were mixed in 96-well plates and incubated in triplicate for 0 and 2 h in a humidified CO_2 static incubator at 37°C. Incubations were stopped by adding 120 μL ice-cold acetonitrile to each well, followed by 5-min sonication and centrifugation at 15,000 × g for 10 min at 4°C. 200 μL of the supernatant was dried using a vacuum concentrator, and the residue was reconstituted with 100 μL of 10% methanol. Aliquot (5 μL) was injected into LC-HRMS system. Extracted ion chromatograms of each metabolite were generated by allowing 5 ppm error from the theoretical mass. Mass Frontier software (version 7.0; HighChem Ltd., Bratislava, Slovakia) was used for the in silico generation of the fragment ions.

***In vitro* metabolism of methallylescaline in human cDNA-expressed CYPs**

The major CYP enzymes responsible for the metabolism of methallylescaline were determined using reaction mixtures containing 10 μL of 11 different human cDNA-expressed CYP enzymes (CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 2J2, 3A4, and 3A5; 40 pmol), 10 μL of methallylescaline stock solution (50 μM), 4 μL of 250 mM magnesium chloride, and 71 μL of potassium phosphate buffer (pH 7.4; 50 mM) in a total incubation volume of 95 μL. The reaction was initiated by adding 5 μL NADPH-generating system, and the mixtures were incubated in triplicate for 1 h at 37°C in a shaking water bath. The reaction was stopped by adding 100 μL ice-cold methanol containing 100 ng/mL homoegonol (IS). The mixtures were centrifuged at 10,000 × *g* for 4 min at 4°C and 150 μL supernatant was evaporated under a N_2 gas stream. The residue was dissolved in 50 mL 45% methanol and a 5 μL aliquot was analyzed using LC-MS/MS. The formation rates of each metabolite were calculated using peak area ratio of the metabolite to homoegonol (IS). Owing to the absence of authentic standards of methallylescaline metabolites, the amounts of produced metabolites were determined using the calibration curve of methallylescaline.

LC-HRMS analysis

A Nexera-X2 UPLC system (Shimadzu, Kyoto, Japan) linked to a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used. Chromatographic separation was performed on a Halo C18 column (2.1 × 100 mm, 2.7 μm; Advanced Materials Technology, Wilmington, DE, USA) by gradient elution of 5% methanol in 0.1% formic acid (A) and 95% methanol in 0.1% formic acid (B) at flow rate of 0.4 mL/min: 40% B for 0–1 min, 40%–60% B for 1–17 min, 60%–95% B for 17–26 min, 95% B for 26–29 min, 95%–40% B for 29–29.2 min, and 40% B for 29.2–32 min. The autosampler and column were held at 4°C and 40°C, respectively.

Mass spectra were acquired using a heated electrospray ionization (HESI) source operating in the positive mode. The optimum HESI source conditions for metabolite profiling were as follows: sheath gas flow rate, 35

Table 1. Retention times, molecular formulae, exact molecular ion masses, mass errors, product ions, and biotransformation of methallylescaline and its metabolites

ID	RT (min)	Formula	Exact Mass ($[M+H]^+$)	Error (ppm)	Product Ions	Biotransformation
Methallylescaline	7.6	$C_{14}H_{21}NO_3$	252.1594	-2.0	235.1329, 203.1068, 181.0861, 167.0703	-
M1	2.5	$C_{14}H_{21}NO_4$	268.1543	-1.1	251.1277, 193.0860, 181.0859, 151.0753	hydroxylation
M2	3.5	$C_{14}H_{21}NO_4$	268.1543	-1.5	251.1277, 193.0860, 181.0859, 151.0753	hydroxylation
M3	4.5	$C_{14}H_{21}NO_4$	268.1543	-1.9	251.1276, 193.0860, 181.0858, 151.0753	hydroxylation
M4	4.6	$C_{14}H_{21}NO_5$	284.1492	-1.8	267.1226, 237.1121, 217.0860, 181.0860	dihydroxylation

(arbitrary units); auxiliary gas flow rate, 10 (arbitrary units); spray voltage, 4 kV; and heater temperature, 350°C. MS data were acquired and processed using Xcalibur software (Thermo Fisher Scientific Inc.). Full MS spectra were obtained from *m/z* 100 to 1500, with a resolution of 70,000, whereas data-dependent MS/MS spectra were acquired at a resolution of 35,000 using normalized collision energies at 23 and 28 eV.

Results and Discussion

In vitro metabolic stability and metabolite identification of methallylescaline were investigated using human hepatocytes.

Approximately 85% of methallylescaline was remained after incubation of methallylescaline with human hepatocyte suspensions at 37°C for 3 h. The $t_{1/2}$, intrinsic clearance (Cl_{int}), and hepatic extraction ratio of methallylescaline were 1113.2 min, 2.0 mL/min/kg, and 9.7% by well-stirred model, respectively, suggesting that the metabolism of methallylescaline may be low and comparable to the poor metabolism of mescaline.[14]

After 2 h incubation of methallylescaline with human hepatocytes, unchanged methallylescaline and four metabolites were detected. Representative extracted ion chromatograms of methallylescaline and its metabolites are shown in Figure 1. The retention times, molecular formulae, theoretical molecular ions $[M+H]^+$, mass errors, product ions, and biotransformation are listed in Table 1.

Methallylescaline produced $[M+H]^+$ ion at *m/z* 252.1594 and the characteristic product ions at *m/z* 235.1329 (a loss of NH_3 from $[M+H]^+$ ion), *m/z* 203.1068 (a loss of CH_3OH from *m/z* 235.1329), *m/z* 181.0859 (a loss of 2-methylallyl group from *m/z* 235.1329), and *m/z* 167.0703 (loss of CH_3NH_2 and 2-methylallyl groups from $[M+H]^+$ ion) (Figure 2A).

M1-M3 showed the $[M+H]^+$ ion at *m/z* 268.1543, 16 amu higher than methallylescaline, indicating hydroxy-methallylescaline (Figure 1). Three metabolites, M1-M3 commonly produced product ions at *m/z* 251.1277 (a loss of NH_3 from $[M+H]^+$ ion), *m/z* 193.0860 (a loss of CH_2CH_2 and CH_2O from *m/z* 251.1277), *m/z* 181.0859 (a loss of hydroxy-2-methylallyl group from *m/z* 251.1277), and *m/z* 151.0753 (loss of hydroxy-2-methylallyloxy and CH_3NH_2 group from $[M+H]^+$ ion), indicating hydroxylation at 2-methylallyl moiety (Figure 2B). However, the accurate hydroxylation sites of M1-M3 could not be identified devoid of the authentic standards.

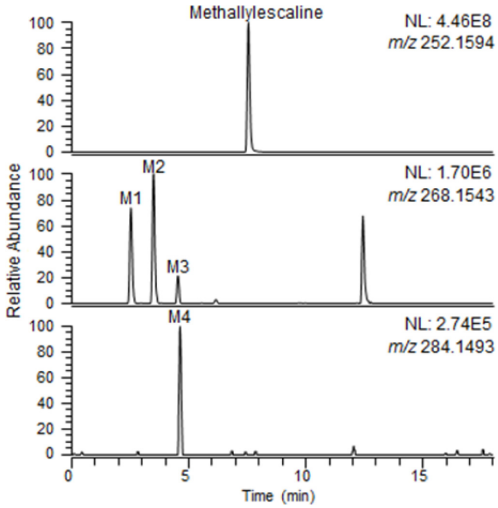

Figure 1. Representative extracted ion chromatograms of methallylescaline and its metabolites identified in human hepatocytes after 2 h incubation at 37°C in a CO_2 incubator.

M4 showed the $[M+H]^+$ ion at *m/z* 284.1492, 32 amu higher than methallylescaline, suggesting dihydroxy-methallylescaline (Figure 1). M4 produced the characteristic product ion at *m/z* 267.1226 (a loss of NH_3 from $[M+H]^+$ ion), *m/z* 237.1121 (a loss of CH_3NH_2 and O from $[M+H]^+$ ion), *m/z* 217.0859 (a loss of CH_3OH and H_2O from *m/z* 267.1226), and *m/z* 181.0859 (a loss of dihydroxy-2-methylallyl group from *m/z* 267.1226), indicating dihydroxylation at 2-methylallyl moiety (Figure 2C).

Methallylescaline was metabolized into four phase I metabolites such as three hydroxy-methallylescaline (M1-M3) and dihydroxy-methallylescaline (M4) via hydroxylation in human hepatocytes (Figure 3). However, the oxidation of amine group to carboxylic acid, reported as the major metabolic pathway of mescaline and 4-bromo-2,5-dimethoxy-phenethylamine,[14,15] and phase II metabolism were not identified in the metabolism of methallylescaline.

To characterize CYP enzymes responsible for the

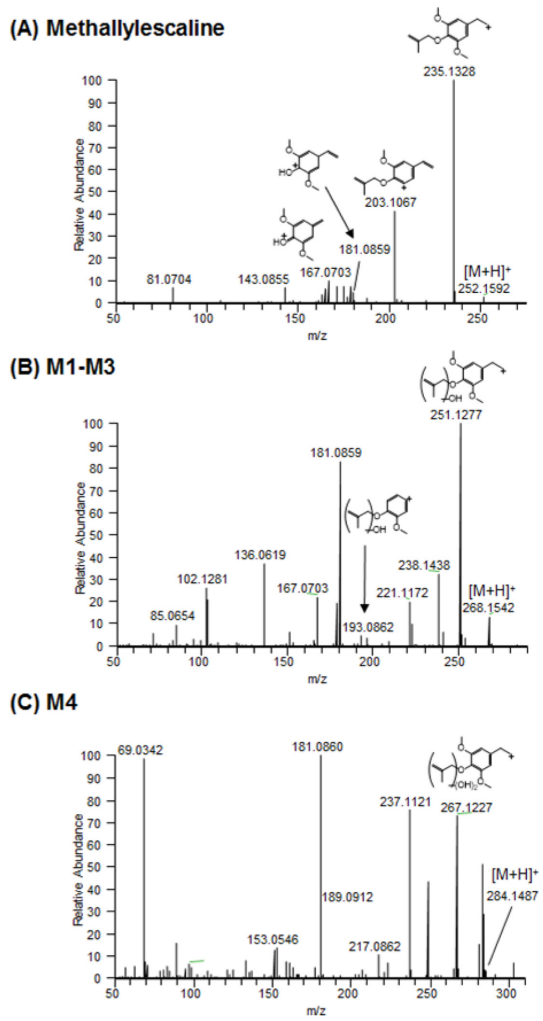

Figure 2. MS/MS spectra of methallylescaline and metabolites identified in human hepatocytes after 2 h incubation at 37°C in a CO_2 incubator. (A) methallylescaline; (B) hydroxy-methallylescaline (M1-M3); and (C) dihydroxy-methallylescaline (M4).

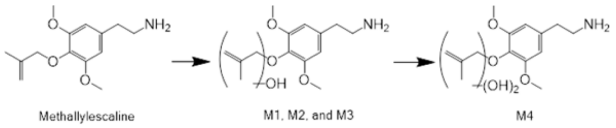

Figure 3. Proposed metabolic pathways of methallylescaline in human hepatocytes

metabolism of methallylescaline, 5 μM methallylescaline was incubated with major 11 human cDNA-expressed CYP enzymes in the presence of NADPH. CYP2D6, CYP2J2, CYP1A2, and CYP3A4 enzymes were responsible for the formation of M1-M3 from methallylescaline, and the formation of M4 was mediated by CYP1A2 and CYP2D6 enzymes (Figure 4).

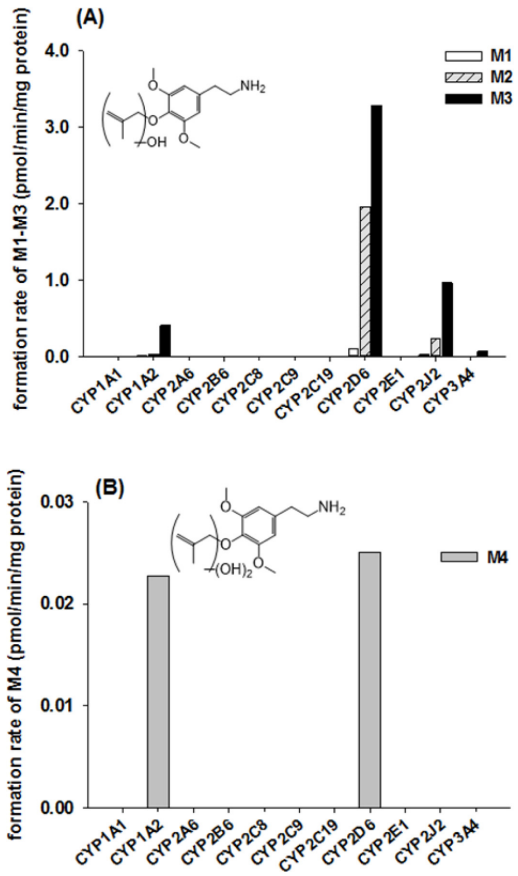

Figure 4. Formation rates of (A) hydroxymethallylescaline (M1-M3) and (B) dihydroxymethallylescaline (M4) obtained from incubation of methallylescaline with human cDNA-expressed CYP enzymes in the presence of NADPH (n=2). The amounts of metabolites were calculated using the calibration curve of methallylescaline.

Conclusions

The hepatic extraction ratio of methallylescaline was 9.7% using well-stirred model in human hepatocytes, suggesting that methallylescaline could be *in vivo* monitored by itself due to low clearance compound. Methallylescaline was metabolized to hydroxymethallylescaline (M1-M3) and dihydroxymethallyl- escaline (M4) via hydroxylation at 2-methylallyl moiety in human hepatocytes. CYP2D6, CYP2J2, CYP1A2, and CYP3A4 enzymes were responsible for the metabolism of methallylescaline.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant, funded by the Korean Government (MSIT) (NRF-2015M3A9E1028325) and the Korea Health Technology R&D Project through the Korea

Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI12C1852).

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