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DISPOSITION AND METABOLISM OF [^{14}C]BRASOFENSINE IN RATS, MONKEYS AND HUMANS

M. ZHU, D.B. WHIGAN S.Y. CHANG AND R.C. DOCKENS

Departments of Pharmaceutical Candidate Optimization (M.Z., D.W., S.C.) and Clinical Discovery (R.D.), Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ

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Author name: Randy C. Dockens, Ph.D.

Address: Bristol-Myers Squibb

Rt 206 & Province Line Road

Princeton, NJ 08543-4000

Phone: (609) 252-5242

Fax: (609) 252-7822

E-mail: randy.dockens@bms.com

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Non-standard abbreviations:

E, brasofensine

ODME, *O*-desmethyl brasofensine

ODMZ, *O*-desmethyl BMS-205912

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NDME, *N*-desmethyl brasofensine

LE, Long Evans

BMS, Bristol-Myers Squibb

BDC, bile-duct cannulated

mM, millimolar

po, oral

iv, intravenous

K, potassium

EDTA, ethylenediaminetetraacetic acid

LC, liquid chromatography

MS, mass spectrometry

TSQ, triple-stage quadrupole

PE, Perkin-Elmer

API, atmospheric pressure ionization

QC, quality control

LLQ, lower limit of quantitation

CSF, cerebrospinal fluid

UV, ultraviolet

LCQ, liquid chromatography quadrupole

vs, versus

$AUC_{(INF)}$, area under the plasma concentration-time curve from 0 to infinity

$t_{½}$, terminal elimination half-life

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C_{max}, maximum plasma concentration

t_{max}, time to maximum concentration

CL_T, total body clearance

Vd_{ss}, volume of distribution at steady-state

AUMC, area under the first moment curve

F, absolute biovailability

$AUC_{(0-T)}$, area under the plasma concentration-time curve from 0 to T, the last measurable time point

amu, atomic mass unit

NADPH, nicotinamide adenine dinucleotide phosphate, reduced form

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ABSTRACT:

Brasofensine is an inhibitor of the synaptic dopamine transporter. It is a geometric isomer of the E-form; the Z-isomer is denoted as BMS-205912. These studies were conducted to characterize the pharmacokinetics, absolute bioavailability, disposition, and metabolism of brasofensine after intravenous (iv) and/or oral (po) administrations of [^{14}C]brasofensine in rats (1.5 mg/kg, iv; 4 mg/kg, po) and monkeys (4 mg, iv; 12 mg, po), and humans (50 mg, po). Brasofensine was rapidly absorbed after oral administration in rats and monkeys, with peak plasma concentrations occurring 0.5-1 hr, but 3-8 hr for brasofensine in humans. Plasma terminal elimination half-lives were ~2 hr in rats, ~4 hr in monkeys, but ~24 hr in humans. Total body clearance and steady-state volume of distribution values were 199 mL/min/kg and 24 L/kg, respectively, in the rat and 32 mL/min/kg and 46 L/kg, respectively, in the monkey. Absolute bioavailability was 7% in rats and 0.8% in monkeys. After a single oral dose, urinary excretion of radioactivity accounted for 20% of the administered dose in rats, 70% in monkeys, and 86% in humans, with the remainder excreted into the feces. Brasofensine had extensive first-pass metabolism following oral administration in humans, monkeys, and rats. It primarily underwent *O*- and *N*-demethylation and isomerization. Some of the desmethyl metabolites were further converted to glucuronides. These primary metabolites and glucuronides of demethyl brasofensine (M1 and M2) were major circulating metabolites in humans, and were also observed in rat and monkey plasma.

Introduction

Brasofensine (BMS-204756, (+)-(*E*)-(1*R*,2*R*,3*S*)-3-(3,4-dichlorophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carbaldehyde *O*-methyloxime) is a novel inhibitor of the synaptic dopamine transporter and has potential use in Parkinson's disease (Johnston, 2004), a disease estimated to affect approximately 500,000 persons in the United States alone (Brooks, 1997; Battistin, 1996). It is known that nigro-striatal dopamine loss is the major factor in Parkinson's disease pathogenesis, with clinical symptoms of Parkinsonism beginning when there is approximately 80% depletion of the striatal dopamine (Kish, 1988). Over time, those with Parkinson's disease have a degeneration of the dopaminergic neurons causing a continued depletion of the amount of dopamine released. This exaggerates the symptoms of Parkinsonism and it is thought that this progression of symptoms could be slowed by inhibition of the dopamine transporter (Mouradian, 1987). The dopamine transporter actively transports released dopamine from the synaptic cleft back into the presynaptic nerve terminal where it is metabolized by monoamine oxidase or extracellularly by catechol-*O*-methyltransferase. Inhibition of the dopamine transporter (dopamine reuptake inhibition) enhances the effectiveness of the diminishing amounts of dopamine that are released into the synaptic cleft. The primary role of brasofensine is thus to conserve the greatly reduced level of endogenous dopamine within the synaptic cleft and also to extend the potency and duration of action of exogenous dopamine from the administration of levodopa. This appears to be true in a marmoset model of Parkinson's disease (Pearce, 2002) but has not yet been demonstrated in the clinic (Frackiewicz, 2002).

The main objectives of these studies were to (*i*) assess the pharmacokinetics and absolute bioavailabilityof brasofensine in rats, monkeys and humans, (*ii*) investigate biotransformation of brasofensine in rats and monkeys and (*iii*) compare the disposition and plasma metabolite profiles of brasofensine in rats, monkeys, and humans after oral and/or iv administrations of radiolabeled brasofensine.

Methods

Experimental Procedures

Chemicals. Radiolabeled [^{14}C]brasofensine (fig. 1), as the maleate salt, had a radiochemical purity of 97.6% and specific activity of 50 μCi/mg free base. [^{14}C]Brasofensine, unlabeled brasofensine, unlabeled BMS-205912, and metabolite standards [*O*-desmethyl brasofensine (ODME[1]), *O*-desmethyl BMS-205912 (ODMZ), and *N*-desmethyl brasofensine (NDME)] were synthesized at Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ and New Brunswick, NJ).

Rat study. Male LE rats (173-281 g) obtained from Charles River Laboratories, Inc. were used in this study after approval of the BMS Animal Care and Use Committee. There were 3 control animals (for blank plasma) and 61 rats dosed with [^{14}C]brasofensine. The rats were fasted overnight prior to dose administration and for approximately 4 hours after dosing. The animals had free access to water at all times. Three BDC-rats were used for 24-hr bile collection. Blood and selected tissues were

collected and animals sacrificed via cardiac puncture exsanguination under halothane anesthesia.

The [^{14}C]brasofensine dosing solutions were prepared in 100 mM sodium phosphate buffer (pH 7.0). Each rat received 4 mg/kg (20 μCi) of the dosing solution by oral (po) gavage or 1.5 mg/kg (20 μCi) iv, administered as a bolus injection in the tail vein. There were three rats per sampling time except at 168 hr postdose where 5 rats were additionally used for collection of urine and feces. Blood samples (one per rat) were collected at 3 (iv only) and 30 minutes, and 1, 3, 6, 12, 24, 48, 96 and 168 hours after dosing. Blood (2-10 mL) was collected at time of sacrifice *via* cardiac puncture into tubes containing K_3EDTA. Urine and feces were collected quantitatively prior to dosing and then daily for up to 7 days after dosing. Plasma was prepared from blood by centrifugation. Plasma, urine, and feces were stored at or below -20˚C until analyzed.

Monkey study. Four male cynomolgus monkeys (5 to 6 kg) were used in this study after approval of the BMS Animal Care and Use Committee. The animals, with indwelling vascular catheters for blood sample collection, were fasted overnight and for 4 hr after dosing, chaired for 12 hr after dosing, and then housed individually in metabolic cages for the remainder of the study. Each monkey was dosed both iv and orally with [^{14}C]brasofensine in a non-randomized cross-over design with a two-week washout period between dosings.

The [^{14}C]brasofensine dosing solutions were prepared in 100 mM sodium phosphate buffer (pH 7.0). Each monkey received 12 mg (50 μCi) by oral gavage and 4 mg (50 μCi) by a 6-minute intravenous infusion into the venous port using an infusion pump. Blood samples were collected prior to dosing and at 6 (iv only), 10 (iv only), 15, 30, and 45 (iv only) min, and 1, 1.5 (po only), 2, 3, 6, 8, 12, 24, 48, 72, 96, 120, 144, and 168 hours (or until the level of radioactivity became less than or equal to twice the background radioactivity count). Blood (1.5 mL) was collected *via* the indwelling vascular catheters into tubes containing K_3EDTA. Urine and feces were collected quantitatively prior to dosing and then daily for up to 7 days after dosing. Plasma was prepared from blood by centrifugation. Plasma, urine, and feces were stored at or below -20˚C until analyzed.

Human study. The study was conducted as a single dose in 6 healthy male subjects. Each subject received 2 mg (2 mL, 100 μCi) of [^{14}C]brasofensine as a solution in water, administered orally following a 10-hr fast. The subjects continued to fast until 4 hr after dosing, at which time they were served lunch.

The study protocol was approved by the Institutional Review Board and Radiation Safety Committee at the investigational site. All subjects gave consent to participate in the study by signing and dating an informed consent form after the study was completely explained to each person.

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All subjects were in good health based on medical history, prestudy physical examinations, and clinical laboratory testing. The mean ± standard deviation age of all subjects who entered the study was 30±8 years, with a range of 22-44 years.

Serial blood samples (7 mL) were collected at predose, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 16, 24, 30, 36, 48, 72, 96, 120, 144, 168, 192, 216, and 240 hr post dose. Immediately after collection, each blood sample was gently inverted a few times for complete mixing with the anticoagulant (K_3EDTA) and placed in chipped ice. Within 1 hr of collection, the blood sample was centrifuged to obtain plasma. The plasma was divided into two portions: one for total radioactivity measurement and the remainder for analysis of unchanged brasofensine and BMS-205912 using a specific LC/MS assay. Blood and plasma were stored at or below -20°C until analyzed. Urine and feces were collected predose, 0-4, 4-8, 8-12, 12-24, and then daily for up to 22 days post dose. An aliquot of each urine and fecal homogenate sample was set aside for total radioactivity measurement. All urine and feces samples were stored at or below -20°C until analyzed.

Analytical Methods.

Brasofensine and BMS-205912. *Rat plasma and urine, monkey plasma and urine, human plasma and urine using liquid chromatography with mass spectrometry (LC/MS)*. The methods involved the addition of internal standard, (BMS-217380 for human plasma and urine; BMS-180448 for rat and monkey plasma; BMS-181101 for rat and monkey urine), to 0.5 mL of human plasma and urine, 0.2 mL of rat plasma and urine, and

monkey plasma, and 1.0 mL of monkey urine. For human plasma and urine, rat urine, and monkey urine the sample and internal standard were mixed with a 0.1 M sodium carbonate solution, hexanes added (for rat plasma and monkey plasma, the extracting solvent was methyl t-butyl ether), shaken for 40 min, and then centrifuged. The tube was placed in a dry-ice/acetone bath, the lower layer frozen, the supernatant decanted and evaporated to dryness at 30-35 ˚C under nitrogen. The residue was reconstituted with a 50/50 mixture of methanol and 0.01 M ammonium acetate solution, pH 6.0, centrifuged, and injected into the chromatographic system. Peak separation was achieved on a YMC basic, 5μ, 2.1 x 100 mm, analytical column (Waters, Milford, MA) at 40 ˚C. For rat urine and monkey urine, detection was done on a SCIEX API-1 mass spectrometer (Applied Biosystems/MDS-Sciex, Foster City, CA) which was operated in the positive electrospray mode. The protonated $[M+H]^+$ ions at *m/z* 327 for brasofensine and BMS-205912, and *m/z* 370 for the internal standard BMS-181101 were monitored for quantitation. The isocratic mobile phase (0.3 mL/min) consisted of 47% methanol and 53% aqueous 0.01 M formic acid. The retention times were about 2.3, 2.5, and 2.6 minutes for BMS-205912, internal standard BMS-181101, and brasofensine, respectively. For human plasma, human urine, rat plasma and monkey plasma, positive ion electrospray LC-MS/MS was used for detection and quantitation with a Finnigan TSQ 7000 mass spectrometer (Thermo-Electron Corp., San Jose, CA) equipped with an electrospray ionization (ESI) source. The mass spectrometer was programmed to admit the protonated molecules $[M+H]^+$ at *m/z* 327 (brasofensine and BMS-205912), *m/z* 396 (BMS-180448 internal standard for rat and monkey plasma), and *m/z* 284 (BMS-217380

internal standard for human plasma and urine) via the first quadrupole (Q1) with collision-induced fragmentation in Q2 (argon collision gas) and monitoring via the third quadrupole (Q3), the product ions at *m/z* 97 for brasofensine and BMS-205912, *m/z* 194 for the BMS-180448 internal standard and *m/z* 100 for the BMS-217380 internal standard. A linear gradient mobile phase was used starting at 28% acetonitrile and 72% Solvent A (75% aqueous 0.01 M ammonium acetate/25% methanol) to 60% acetonitrile and 40% Solvent A in 2 minutes and holding this mixture for 1 min. The flow rate was 0.3 mL/min. The retention times were about 1.4, 2.0, 2.1, and 5.0 minutes for BMS-205912, brasofensine, BMS-217380, and BMS-180448, respectively.

Using peak area ratios, standard curves were obtained by quadratic regression weighted by $1/x^2$. Predicted concentrations were calculated using the regressed equation. Spiked QC samples were prepared, before the initiation of the study, in control plasma and urine using reference standards for brasofensine and BMS-205912, and stored with the study samples. QC samples were analyzed with study samples to establish stability and monitor acceptability of an analytical run.

The analytical runs were accepted on the basis of *a priori* acceptance criteria as follows:

1. The back-calculated concentrations of at least three-fourths of all calibration standards were to be within ±15% of their individual nominal concentrations (±20% at the LLQ).

2. At least one replicate of the lowest concentration in the standard curve was to be within ±20% of the nominal concentration for that level to qualify as the LLQ. If this criterion was not met, the next level was subjected to the same test and the LLQ raised accordingly.

3. The back-calculated concentrations of all analytical QC samples were to be within ±15% of their individual nominal concentrations, with at least one acceptable QC sample at each level.

The plasma standard curve range for brasofensine and BMS-205912 was 0.02 to 5 ng/mL in human plasma and urine, 0.05 to 10 ng/mL in rat and monkey plasma, 0.1 to 5 ng/mL in monkey urine, and 0.25 to 25 ng/mL in rat urine. The lowest end of the standard curve range represented the LLQ for brasofensine and BMS-205912. During analysis of study samples, the mean observed concentrations of the QC samples were within ±7% from nominal values. The between- and within-day coefficient of variations (CV) were within 14%. These results indicated that the assay methods were precise, accurate, and reproducible for the analyses of brasofensine and BMS-205912 in human plasma and urine, rat plasma and urine, and monkey plasma and urine. Brasofensine and BMS-205912 were stable under the assay conditions.

Total Radioactivity. Total radioactivity in urine samples (1 mL of urine added to 15 mL of Hionic-Fluor) was measured without any processing. Fecal samples were first homogenized with approximately 3 volumes of 100 mM phosphate buffer or water and combusted for measurement of total radioactivity.

Samples of the following rat tissues or fluids were solubilized in the scintillation cocktail directly, or first combusted and then analyzed using liquid scintillation counting: adrenal glands, bladder, blood, bone, bone marrow (femur), brain, CSF, eyes, heart, kidneys, large intestine, large intestinal contents, liver, lungs, muscle (thigh), plasma, skin (nonpigmented and pigmented), small intestine, small intestinal contents, spleen, stomach, stomach contents, testes, thyroid, carcass and urine. The total organ or a tissue sample was accurately weighed and digested with an appropriate amount of 1N sodium hydroxide. An accurately weighed amount of the digested sample was then mixed with 15 mL of Ultima-Gold scintillation cocktail. All sample combustions were done in a Model 306 or 307 Sample Oxidizer (Packard Instrument Company).

Metabolite Profiling. For metabolite profiling, a portion (0.45 - 1.0 mL) of the pooled plasma or urine sample was applied to an activated Oasis Extraction Cartridge (Waters Associates, Milford, MA). The cartridge was eluted with 2 mL of water followed by 2 mL of methanol. The methanol fractions were dried under nitrogen and reconstituted with 0.5 mL of methanol (20%)/water (80%). The extraction efficiency of samples prepared for metabolic profiling was greater than 90%.

For plasma metabolite profiling by off-line HPLC/liquid scintillation counting, two reverse phase HPLC systems were utilized. One (method A) used a PKB-100 column [Supelco (Sigma-Aldrich, St. Louis, MO); 4.6 x 250 mm] and a linear stepwise gradient system with Solvent A [0.06% trifluoroacetic acid (TFA) in water, adjusted to pH 3.0 with NH_4OH] and Solvent B (methanol). Solvent B in the gradient started at 27% and then increased as follows: 42% (30 min), 70% (35 min), 75% (40 min), 80% (50 min), and 90% (55 min). The second HPLC system (method B) used a Zorbax Rx-C_{18} column (MAC-MOD Analytical, Chadds Ford, PA; 4.6 x 250 mm) and a linear stepwise gradient systems with Solvent A (0.06% TFA in water, pH 3.0) and solvent B (acetonitrile). Solvent B in the gradient was held at 27% for the first 3 min and then increased as follows: 35% (35 min), 70% (45 min), maintained at 70% (45-47 min), 90% (49 min) and maintained at 90% (49 to 55 min). HPLC analyses were performed on a Shimadzu Class VP System equipped with two pumps (model CL-10AT), an autoinjector (SIL 10AD) and a diode array detector (SPD-M10A) at a flow rate of 1 mL/min. The UV absorption of the effluent was monitored at a wavelength of 216 nm. The HPLC eluate was collected by a fraction collector (0.5 min/tube). Each fraction was mixed with 4.5 mL of Exolite liquid scintillation cocktail and counted for 20 - 60 min in a Packard liquid scintillation analyzer (Perkin-Elmer, Waltham, MA). For urine metabolite profiling, HPLC method A was used and radioactivity in the HPLC eluate was determined with an on-line radioactivity detector (β-Ram IN/US Systems, Tampa, FL). HPLC eluate was mixed with IN-Flow™ 3

cocktail (IN/US System), at a ratio of 1 to 3, and the mixture was passed through a 500 µl liquid detection cell.

Metabolite Identification. Radioactive metabolites in rat and monkey urine were identified by HPLC/radio-flow detection/MS. HPLC eluate (method A) from the diode array detector was split and transferred into a radioflow detector (0.8 mL/min) and an ion trap MS (0.2 mL/min) (LCQ, Finnigan). Mass spectrometric analysis was conducted using electrospray positive mode at a capillary temperature of 200 °C. MS^n (n=1-3) spectra of metabolites were obtained with collision energies of 15-20 electron volts. Plasma metabolites were identified by comparison of their HPLC retention times with those of reference standards and the metabolites identified in rat urine. If the retention times of an unknown metabolite in radiochromatograms from analyses by both HPLC methods (PKB and Zorbax) were the same as those of a reference standard or a known urinary metabolite, the structure of the unknown metabolite was assigned.

Pharmacokinetic Analysis. Plasma concentrations *vs.* time data for brasofensine and BMS-205912 were analyzed by noncompartmental methods (Gibaldi and Perrier, 1982). The terminal log-linear phase of the plasma concentration *vs.* time curve was identified by least squares linear regression of data points that yielded a minimum mean square error. The $AUC_{(INF)}$ was determined by a combination of trapezoidal and log-trapezoidal methods, plus the extrapolated area. The extrapolated area was determined by dividing the observed concentration at the time of the last nonzero plasma concentration by the

slope (β) of the terminal log-linear phase. The $t_{½}$ of the terminal log-linear phase was calculated as ln(2) divided by the absolute value of β. The peak plasma concentration, C_{max}, and the time at which C_{max} occurred, t_{max}, were obtained from the observed data. Total body clearance (CL_T) and steady-state volume of distribution (Vd_{ss}) for brasofensine were calculated as follows:

$$CL_T = Dose(iv)/AUC_{(INF)}$$

and

$$Vd_{ss} = Dose(iv) \bullet AUMC/(AUC_{(INF)})^2,$$

where AUMC is the area under the first moment of plasma concentration *vs.* time curve. The absolute bioavailability (F) of brasofensine and BMS-205912 was estimated as a ratio of the plasma $AUC_{(INF)}$ of unchanged brasofensine or BMS-205912 after oral to that after iv administration. Mean residence time (MRT) was calculated as $AUMC/AUC_{(INF)}$.

Results

Excretion of Radioactivity. Mean (standard deviation) percentage recovery of total radioactivity in urine and feces of rats, monkeys, and humans is summarized in Table 1. In all species studied, The percentage of administered radioactivity recovered was comparable between iv and oral administration. After either iv or oral dosing in rats and monkeys, approximately 20% and 70% of total radioactivity, respectively, was recovered in the urine. Fecal excretion accounted for approximately 70% and 20% in the rat and

monkey, respectively. The majority of the administered dose was recovered in the first 24 hr post dose. Overall, approximately 90% of the total iv and oral dose was excreted up to 168 hr post dose in the rat and monkey. Biliary excretion of total radioactivity in three BDC rats after oral dosing of [^{14}C]brasofensine accounted for 64% of the total dose in 24 hr.

In humans, the mean cumulative excretion of total radioactivity in urine over 22 days post dose was 86% following oral dosing of [^{14}C]brasofensine. Approximately 90% of the radioactive dose recovered in urine was excreted over the first week post dose. Fecal excretion in the human accounted for 11% of the oral dose. Overall, 97% of the total oral radioactivity administered was recovered in urine and feces over 22 days post oral dosing.

Plasma Time Course of Total Radioactivity and Pharmacokinetics of Brasofensine. Mean (standard deviation) plasma concentration-time profiles of total radioactivity, brasofensine and BMS-205912 after iv and oral dosing of brasofensine in rats and monkeys and after oral dosing in humans are shown in fig. 2-4. Mean (standard deviation) pharmacokinetic parameters of total radioactivity, and brasofensine and BMS-205912, for rats, monkeys and humans are summarized in tables 2 and 3, respectively.

The AUC values of total radioactivity, compared to the AUC values of brasofensine following [^{14}C]brasofensine administration were 49-fold and 628-fold greater following iv and po dosing, respectively, in the rat, 8-fold and 1123-fold greater following iv and po dosing, respectively, in the monkey, and 13-fold following po dosing in humans. The half-life values of total radioactivity were comparable following iv and oral administration. The concentrations of brasofensine and BMS-205912 decreased much more rapidly than those for radioactivity. The exposure to BMS-205912 was <6% of the brasofensine exposure following administration of brasofensine in the rat and monkey. In contrast, the exposure to BMS-205912 in humans following oral brasofensine was approximately 90% of the brasofensine exposure with mean BMS-205912 concentrations increasing more gradually than those of brasofensine, and then exceeding brasofensine concentrations after 72 hours post dose.

In the rat, brasofensine $AUC_{(INF)}$ values were 125 ng·hr/mL after iv dosing (1.5 mg/kg) and 25 ng·hr/mL after oral dosing (4 mg/kg), with an absolute bioavailability of 7% (Table 3). The corresponding exposure to BMS-205912 was approximately 1 ng·hr/mL following both routes of brasofensine administration. After oral dosing, brasofensine was absorbed rapidly with peak concentrations of 11 ng/mL achieved within 1 hr of dosing. The decline in plasma concentrations yielded a terminal plasma half-life of 1.7-3.5 hr. Mean residence time was 2.1-2.5 hr. Total body clearance of brasofensine in rats was 199 mL/min/kg and steady-state volume of distribution was 24 L/kg.

In the monkey, mean brasofensine $AUC_{(INF)}$ values were 507 ng·hr/mL after iv dosing (4 mg) and 9 ng·hr/mL after oral dosing (12 mg), with a mean absolute bioavailability of 0.8%. The corresponding exposure to BMS-205912 was approximately 20 ng·hr/mL following iv brasofensine administration, but was undetectable following oral brasofensine administration. After oral dosing, brasofensine was absorbed rapidly with a peak concentration of 3.3 ng/mL achieved within 1 hr of dosing. The decline in plasma concentrations yielded a mean terminal plasma half-life of 3.9 hr. Mean residence time was 5.4 hr. Mean total body clearance of brasofensine in monkeys was 32 mL/min/kg and mean steady-state volume of distribution was 46 L/kg.

In humans, mean $AUC_{(INF)}$ values were 39.0 ng·hr/mL and 35.5 ng·hr/mL for brasofensine and BMS-205912, respectively, after oral dosing (50 mg) of brasofensine. After oral dosing, brasofensine was absorbed relatively slowly with a peak concentration of 1.2 ng/mL achieved within 7 hr of dosing. The peak concentration for BMS-205912 following brasofensine administration was approximately one-third the brasofensine concentration and occurred much later (~24 hr). The decline in plasma concentrations yielded a mean terminal plasma half-life of 24 hr and 55 hr for brasofensine and BMS-205912, respectively. Mean residence times were 39 and 96 hr, respectively.

Distribution of Radioactivity in Tissues and Fluids. Figure 5 depicts the concentrations of total radioactivity in selected tissues in LE rats after iv and oral administrations of brasofensine. The tissues selected represent organs associated with

absorption (stomach and small intestine), organs associated with elimination (kidneys, liver, and large intestine), organ associated with site of action (brain) and another organs that showed significant concentrations of radioactivity at the last time of measurement (eye, lung, adrenal gland, spleen and blood).

Highest concentrations of radioactivity after either oral or iv dosing were observed at 1 hr post dose in most tissues except for the eyes and small intestine following iv administration where peak concentrations occurred at 3 hr post dose, and except for the eyes, small intestine and large intestine following oral dosing where peak concentrations occurred at 12, 3 and 12 hr post dose, respectively. Except for CSF, all other tissues had higher concentrations of total radioactivity than plasma for at least the first 12 hours. All tissues, except CSF, had detectable concentrations at 168 hr post dose. The decline of radioactivity in plasma and blood were almost superimposible until around 48 hours when the radioactivity in blood started to decline less than the decline in plasma. The decline in concentration in most tissues seemed to parallel the decline in blood while some tissues, like the organs of excretion, tended to parallel the decline in plasma.

Metabolite Profiles. HPLC radiochromatograms of rat, monkey, and human plasma are shown in fig.6. There were seven radioactivity peaks in the human plasma profile. Based on their retention times, five of these peaks corresponded to the parent drug (E), its Z-isomer (Z; BMS-205912), the *O*-desmethyl E (ODME) metabolite, the *O*-desmethyl Z (ODMZ) metabolite, and the *N*-desmethyl E (NDME) metabolite (fig 1). Together, these

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five radioactive peaks accounted for 85% of the total radioactivity in plasma (Table 4). Parent drug in human plasma was the predominent component (45%) followed by ODME (20%), ODMZ (11%), Z-isomer of the parent drug (6%), and NDME (3%). The remainder of the radioactivity (15%) was present as two minor metabolite peaks (M1 and M2), identified as glucuronide conjugates of *O*-desmethyl brasofensine and *N*,*O*-didesmethyl brasofensine, respectively (fig 6). All these metabolites in human plasma had the same HPLC retentions as those of the metabolite standards [ODMZ, ODME, Z-isomer (BMS-205912) and NDME] and metabolites identified in rat urine (M1 and M2) by LC/MS/MS.

The plasma metabolite profiles in rats and monkeys were more complex in terms of the number of radioactivity peaks compared to humans. The rat plasma contained all of the metabolites observed in human plasma (19% as ODME, 9% as ODMZ, 2% as NDME, 5% as M1+M2, and 2% as parent compound; see Table 4). The Z-isomer (BMS-205912) in the rat plasma was detected by LC/MS/MS, but not by HPLC/radioactivity analysis due to its low abundance (<2% of the total radioactivity). All human plasma metabolites were also observed in monkey plasma (Table 4 and fig 6). The parent drug and its Z-isomer (BMS-205912) were detected by LC/MS/MS (data not shown), but not by radiochromatographic analysis since each had less than 2% of the total plasma radioactivity. However, the radioactivity distribution in monkey plasma was different from that in human plasma. Human metabolites only accounted for 9% of the total

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radioactivity in monkey plasma, and none of the prominent radioactivity peaks were observed in human plasma.

The metabolite profile of [^{14}C]brasofensine in rat urine is shown in fig. 7A, which was similar to that in rat plasma. M1, M2, ODME and ODMZ were observed as major urinary metabolites. The parent drug and its Z-isomer were not observed in rat urine. The metabolite profile of [^{14}C]brasofensine in monkey is shown in fig. 7B, which was consistent with that in monkey plasma (fig. 6B). The major drug-related components in monkeys were glucuronides of oxidative metabolites, such as O- and N-desmethyl metabolite (Table 5). No brasofensine or Z-isomer was detected in monkey urine (fig. 7B) , suggesting that brasofensine was extensively metabolized in monekys since more than 60% of dose was recovered in the urine (Table 1).

Identification of Urine Metabolites by LC/MS/MS. Brasofensine metabolites in rat and monkey urine was structural characterized uing ion trap LC/MS. Protonated molecules, MS^n spectra (n = 2 and 3) and proposed structures of brasofensine metabolites in monkey urine are summarized in Table 5. The product ion spectrum of brasofensine (E) is shown in fig. 8A. ODME and its geometric isomer ODMZ had the same protonated molecule (MH^+) at *m/z* 313, 14 amu less than that of the parent drug, indicating these two metabolites were formed via demethylation. The product ion spectrum and its interpretation of ODME are shown in fig 8B, which were identical to those of ODMZ

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(data not shown). The product ion at *m/z* 282 from the loss of a CH_3N group (-31) suggests that the metabolite was the *O*-demethylation product. The product ions at *m/z* 295 and 269 were produced from both ODME and the parent drug (fig. 8) indicating that the *N*-methyl group remains in ODME. The identity of ODME and ODMZ were further confirmed by chromatographic and mass spectral comparison with those of the synthetic standards.

M1 had an MH^+ at *m/z* 489 and the most intensive product ion at *m/z* 313 from the loss of 176 amu (fig. 9a), indicating M1 was a glucuronide of a demethylated product. MS^3 (489→313→) spectrum of M1 showed several major fragment ions at *m/z* 295, 282, 266, and 264 (fig. 9B) are identical to that of ODME (fig. 8B). Therefore, M1 was identified as a glucuronide conjugate of *O*-desmethyl brasofensine (or its Z-isomer). Most likely, the glucuronic acid was attached to the oxime oxygen (fig. 9A). M2 had a protonated molecule at *m/z* 475 (fig. 10A). The product ion at *m/z* 299, resulting from a loss of 176 amu, clearly indicates M2 is a glucuronide (fig. 10A). The protonated ion (*m/z* 299) of the aglycone was 28 amu less than that of the parent drug (MH^+=327) suggesting the loss of two methyl groups from brasofensine or its Z-isomer. MS^3 spectrum of M2 (475→299→) displayed product ions at *m/z* 281, 266, 264, 254, and 214 (fig. 10b), consistent with the structure of *O,N*-desmethyl brasofensine (fig. 10). Therefore, M2 is assigned as a glucuronide of *O,N*-didesmethyl brasofensine. Most likely, the glucuronic acid was attached to the oxime group (fig. 10A).

M13 and M5 were major metabolites in monkey plasma (fig. 6B) and urine (fig. 7B), but these metabolites were not presented significantly in human plasma (fig. 6A), rat plasma (fig. 6C) and rat urine (fig. 7A). M13 and M17 had the same protonated molecule at m/z 505 (Table 5). MS2 spectra of these metabolites showed the formation of an ion at m/z 329 resulting from a loss of 176 (fig.11A and Table 5), indicating M13 and M17 (Figure 17) were glucuronide conjugates of brasofensine derivatives. The ion at m/z 329 corresponded to the molecular ion of a metabolite formed by the combination of monohydroxylation (+16) and demethylation (-14). MS^3 spectra (MS3 analysis 505→329→) M13 and M17 were identical (fig. 11B and Table 5), respectively. The observed loss of the OCH_3 group to yield an ion at m/z 297 suggests that a methyl group was attached on the oxygen atom. Therefore, M13 and M17 were identified as glucuronide conjugates of monohydroxylated *N*-desmethyl brasofensine.

M11, M14 and M15 had the same molecular ion at m/z 491, but different HPLC retention times (Fig. 6B). MS^2 analysis of the three metabolites showed the formation of an ion at m/z 315 by the loss of 176, indicating that M11, M14 and M15 were glucuronide conjugates of brasofensine derivatives. The ion at m/z 315 (491-176) corresponded to the protonated aglycone that was formed by the combination of monohydroxylation (+16) and didemethylation (-28). MS^3 spectra of M15 and the interpretation of the fragmentation are displayed in fig, 12. Based on MS^n spectral data M11, M14 and M15 were identified as glucuronide conjugates of monohydroxylated *O*, *N*-didesmethyl brasofensine (Table 5).

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Discussion

From the BDC rats, dosed with [^{14}C]brasofensine, 64% of the administered radioactivity was recovered in bile indicating that at least this amount plus that recovered in the urine was absorbed from the gastrointestinal tract in rats (i.e., at least 85%). Although similar data is not available from the monkey, mean recovery of total radioactivity in urine after oral administration of [^{14}C]brasofensine was comparable to that after iv administration, suggesting almost complete absorption of brasofensine. Also, at least 86% of the dose was absorbed in humans since this percent of dose was recovered in urine following oral administration of brasofensine. Despite this high percentage of administered dose being absorbed, the absolute bioavailability of brasofensine was 7% in the rat and 0.8% in the monkey. Also, the AUC ratio of unchanged drug to total radioactivity was highest in human with a value of 15%. This suggests a significant portion of the orally administered dose is subjected to first-pass metabolism.

Absorption of brasofensine in rat and monkey after oral administration was rapid compared to that in humans (T_{max} of 1 hr *vs* 7 hr). Plasma levels of brasofensine and BMS-205912 in the rat fell more quickly than in the monkey and in a comparable fashion following both iv and oral administration. Plasma concentrations of brasofensine and BMS-205912 in the monkey fell more quickly following oral administration compared to iv administration, probably reflecting the degree of first-pass metabolism. The plasma levels of BMS-205912 following brasofensine administration were one to two orders of

magnitude lower than parent drug. However, in humans, there were significant concentrations of BMS-205912 following oral brasofensine administration yielding a 1:1 AUC ratio.

Brasofensine appears to be extensively distributed into tissues, as indicated by its high volume of distribution in rats (24 L/kg) and monkeys (46 L/kg), compared to that of total body water of 0.7 L/kg in these species (Davies, 1993). Total body clearance of brasofensine was approximately 7-fold higher than the liver plasma flow of 30 mL/min/kg in the rat and 120% of the liver plasma flow of 26 mL/min/kg in the monkey (Davies, 1993), probably reflecting the high degree of extraction into the liver and subsequent metabolism.

After single 4-mg/kg oral and 1.5-mg/kg iv doses of [^{14}C]brasofensine to male LE rats, radioactivity was distributed throughout the body and there was no evidence that radioactivity tended to accumulate in any tissue. The increase in the concentration of radioactivity in the intestinal tract up to 6 hr after iv administration indicated the biliary secretion of the drug. After oral administration, the concentrations in the intestinal tract probably represented any unabsorbed drug and the drug secreted in bile. After oral and iv doses to LE rats, the maximum concentrations of total radioactivity were generally observed at the one or three hour time for all tissues, with the exception of the large and small intestines. Concentrations of total radioactivity were detectable in all of the tissues examined after oral and iv doses at 168 hr after dosing, indicating extensive distribution

of [^{14}C]brasofensine, its metabolites, or both into most tissues. Radioactivity was observed in brain suggesting that brasofensine and/or its metabolites cross the blood-brain barrier to reach its proposed site of action.

In human plasma, brasofensine was the most abundant drug-related component, accounted for 45% of the total plasma radioactivity (Table 4). The metabolites observed in human plasma were ODME, ODMZ, M1, M2, Z, and NDME (fig. 14), which accounted for the rest of the human plasma radioactivity (55%). These human plasma metabolites were also detected in both rat and monkey plasma, although their relative abundance were different across species. Urinary excretion was the major elimination pathways of brasofensine in humans and monkeys, accounting for 86.4% and 64.7% of an oral dose, respectively (Table 1). Brasofensine and its Z-isomer were not detected in human (data not shown) and monkey urine (fig. 7) by HPLC/radio-flow detection, suggesting metabolism played a dominant role in drug clearance in both humans and monkeys. Metabolite profiling of rat urine (21.2% of an oral dose) showed the major drug-related components in rats were metabolites rather than the parent drug and Z-isomer (fig. 6A), indicating that metabolism may significantly contribute to brasofensine clearance in rats.

The metabolite profiles in monkey and rat urine (fig. 7) suggest that brasofensine underwent three primary biotransformation pathways in monkeys and rats (fig. 14): (1) demethylation to ODME from brasofensine and to ODMZ from Z-isomer; (2) *N*-

demethylation of brasofensine to NDME and (3) isomerization of the drug to form Z-isomer (BMS-205912). ODME, NDME and/or didemethylated products further formed glucuronides. Although metabolite profile in human urine was not determined in the present study, human plasma metabolite profile (fig 6A) suggests the similar biotransformation reactions occurred in humans. Additionally, incubations of radiolabeled brasofensine (50 μM) in liver microsomes from humans and liver S9 preparations from humans, rats and monkeys in the presence of NADPH only generated two metabolites, ODME and NDME (data not shown), indicating that the *O*- and *N*-demethylation reactions of brasofensine were primary CYP-mediated metabolic pathways in these species. However, the biochemical mechanism of the brasofensine isomerization remains to be determined.

In summary, a number of differences were observed in the disposition of brasofensine in rats, monkeys and humans. Brasofensine was rapidly absorbed after oral administration in rats and monkeys (~1 hr to reach C_{max}) whereas it took ~7 hr to reach C_{max} following oral administration in humans. [^{14}C]Brasofensine shows extensive distribution into rat tissues. The levels of BMS-205912 following brasofensine administration is one to two orders of magnitude lower than parent compound in the rat and monkey, but are comparable in the human yielding a 1:1 AUC ratio. The terminal plasma half-life of brasofensine following oral administration is 2-4 hours in rats and monkeys but approximately 24 hours in humans. Biliary excretion was the predominant route of elimination in the rat and urine was the predominant route of elimination in the

monkey and in humans. Brasofensine is subjected to significant first-pass metabolism in all three species, which plays a significant role in drug clearance. In humans, monkeys, and rats, brasofensine primarily undergoes isomerization and demethylation biotransformation. Some of the desmethyl metabolites are further converted to glucuronides.

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Footnotes

Send reprint requests to: Dr. Randy Dockens, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 09543-4000.

Legends for Figures

Figure 1. Chemical structures of (a) brasofensine maleate (E-isomer), (b) BMS-205912 (Z-isomer), (c) *O*-desmethyl brasofensine (ODME), (d) *O*-desmethyl BMS-205912 (ODMZ), (e) *N*-desmethyl brasofensine (NDME). The asterisk (*) indicates the position of ^{14}C on brasofensine (all six carbons were labeled).

Figure 2. Composite plasma concentration-time profiles of total radioactivity (-▲-), brasofensine (-●-), and BMS-205912 (-○-) after (a) iv administration (1.5 mg/kg) and (b) oral administration (4 mg/kg) of [^{14}C]brasofensine in LE rats.

Figure 3. Mean (standard deviation) plasma concentration-time profiles of total radioactivity (-▲-), brasofensine (-●-), and BMS-205912 (-○-) after (a) iv administration (4 mg) and (b) oral administration (12 mg) of [^{14}C]brasofensine in cynomolgus monkeys.

Figure 4. Mean (standard deviation) plasma concentration-time profiles of total radioactivity (-▲-),brasofensine (-●-), and BMS-205912 (-○-) after oral administrations (50 mg) of [^{14}C]brasofensine in humans.

Figure 5. Mean concentrations of total radioactivity in selected tissues from LE rats after iv (top) and oral (bottom) administration of [^{14}C]brasofensine

●, large intestine; Δ, small intestine; ○, stomach; ■, liver; ▲, kidney; ♦, plasma; , blood; ▼, brain; □, eye; ◊, lung; +, adrenal gland; +, spleen.

Figure 6. Metabolite profiles of [^{14}C]brasofensine in human (A, pooled from 4-24 h), monkey (B, pooled from 1.5-3 h)and rat (C, 2 h) plasma.

Figure 7. Metabolite profile of brasofensine in rat urine (A, pooled 0-96 h) and monkey (B, pooled 0-24 h).

Figure 8. Product spectra and structures of brasofensine (A) and ODME (B)

Figure 9. Mass spectra and proposed structure of M1. A: Product ion spectrum of M1 ($M+H^+$ = 489). B: MS^3 spectrum (489 → 313) of M1.

Figure 10. Mass spectra and proposed structure of M2. A: Product ion spectrum of M2 ($M+H^+$ = 475). B: MS^3 spectrum (474 → 299) of M2.

Figure 11. Mass spectra and proposed structure of M13. A: Product ion spectrum of M13 ($M+H^+$ = 505). B: MS^3 spectrum (505 → 329) of M13.

Figure 12. Mass spectra and proposed structure of M15. A: Product ion spectrum of M15 ($M+H^+$ = 491). B: MS^3 spectrum (491 → 297) of M15.

Figure. 13. Proposed primary biotransformation pathways of brasofensine in humans, monkeys and rats

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Table 1. Mean (standard deviation) percentage cumulative recovery of total radioactivity[a] postdose after iv and oral administration of [^{14}C]brasofensine in male LE rats (N=5), male cynomolgus monkeys (N=4) and healthy male subjects (N=6).

Species	Matrix	iv Solution	Oral Solution
		Dose: 1.5 mg/kg	Dose: 4 mg/kg
Rat	Urine	22.8 (3.8)	21.2 (2.8)
	Feces	62.1 (3.3)	71.2 (2.7)
	Carcass[b]	5.4 (0.5)	5.1 (0.4)
	Total	90.3 (3.7)	97.5 (0.6)
		Dose: 4 mg	Dose: 12 mg
Monkey	Urine	66.8 (4.0)	64.7 (4.0)
	Feces	23.4 (2.6)	22.7 (3.8)
	Total	90.2 (2.7)	87.4 (2.9)
			Dose: 50 mg
Human	Urine	ND	86.4 (4.5)
	Feces	ND	11.4 (2.4)
	Total	ND	97.7 (2.4)

[a]rat and monkey=7 days postdose; human=22 days postdose

[b]whole animal

ND=not determined

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Table 2. Mean (standard deviation) pharmacokinetic parameters for total radioactivity in LE rats, cynomolgus monkeys and humans after iv and oral administration of [^{14}C]brasofensine

Species	Dose, route	C_{max} (ng-eq/mL)	T_{max}[b] (hr)	$AUC_{(INF)}$[c] (ng-eq•hr/mL)	Terminal Plasma $T_{½}$ (hr)
Rat[a]	1.5 mg/kg, iv	---	---	6143	31
	4 mg/kg, po	344	3	15631	31
Monkey	4 mg, iv	---	---	4254 (764)	52 (27)
	12 mg, po	577 (187)	2	10108 (1968)	88 (40)
Human	50 mg, po	5.4 (2.1)	8	512 (142)	79 (19)

[a]Pharmacokinetic parameters derived from a composite profile

[b]Median

[c]AUC for monkey is $AUC_{(0-T)}$, where T is the last measurable sampling time

Table 3. Mean (standard deviation) pharmacokinetic parameters for brasofensine and BMS-205912 in LE rats, cynomolgus monkeys and humans after iv and oral administration of brasofensine

Species	Dose, route	C_{max} (ng/mL)		T_{max}[c] (hr)	$AUC_{(INF)}$[d] (ng•hr/mL)		Terminal Plasma $T_{½}$ (hr)	MRT (hr)	CL_T (mL/min/kg)	VD_{ss} (L/kg)	F (%)
		brasofensine	BMS-205912		brasofensine	BMS-205912					
Rat[a]	1.5 mg/kg, iv	---	---	---	125	1.04	3.5	2.1	199	24	---
	4 mg/kg, po	10.5	0.57	1	24.9	1.16	1.7	2.5	---	---	7.4
Monkey	4 mg, iv	---	---	---	507 (367)	20.4 (19.3)	NA[e]	NA[g]	32 (17)	46 (5)	---
	12 mg, po	3.3 (4.1)	ND[b]	1	9.0 (8.4)	ND[b]	3.9 (2.2)	5.4 (0.7)	---	---	0.8
Human	50 mg, po	1.2 (0.5)	0.31 (0.11)	7	39.0 (28.5)	35.5 (21.4)	24 (13)[f]	39 (23)[h]	---	---	---

[a]Pharmacokinetic parameters derived from a composite profile
[b]No detectable concentrations
[c]Median
[d]AUC for monkey is $AUC_{(0-T)}$, where T is the last measurable sampling time

[e]the terminal slope could not be determined
[f]Terminal plasma $T_{½}$ for BMS-205912 was 55 (37) hr
[g]AUC(INF) could not be determined
[h]MRT for BMS-205912 was 96 (43) hr

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Table 4. Percentage distribution of brasofensine in human, monkey and rat plasma[(a)]

Species	Radioactivity in plasma corresponding to human metabolites (%)							
	M1	M2	ODMZ	ODME	Z	E	NDME	Total
Human	9	6	11	20	6	45	3	100
Monkey	4		2	3	ND	ND	trace	9
Rat	5		9	19	ND	2	2	37

(a) Radioactivity distribution in plasma was determined by using HPLC method B. The method cannot separate M1 from M2.

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Table 5. Relative percent distribution, mass spectra data, and proposed structures of metabolites of [^{14}C]brasofensine in monkey urine

Metabolite	Percent of radioactivity	MS^n spectral data		Metabolite identity
		$M+H^+$	MS^2 and MS^3 product ions[(d)]	
M1	1	489	**MS^2**: 313, 295, 280, 266. **MS^3 on 313**: 295, 282, 266, 264 and 159.	Glucuronide of *O*-desmethyl brasofensine[(c)]
M2	10	475	**MS^2**: 299, 281, 266. **MS^3 on 299**: 281, 266, 264, 214.	Glucuronide of O,*N*-didesmethyl brasofensine[(c)]
ODME	Trace[(a)]	313	**MS^2**: 295, 282, 269, 264, 159.	*O*-desmethyl brasofensine (BMS-282697)
M6	3	473	**MS^2**: 197	Glucuronide conjugate of a phase I metabolite
M8	6	343	**MS^2**: 325, 295, 278, 268. **MS^3 on 295**: 278, 268, 264, 252, 123.	Monohydroxylated brasofensine[(c)]
M9		505	**MS^2**: 487, 469, 458, 329. **MS^3 on 329**: 311, 298, 282, 280.	Glucuronide of monohydroxylated *O*-desmethyl brasofensine[(c)]
NDME	Trace[(a)]	313	**MS^2**: 296, 281, 255, 159.	N-desmethyl Brasofensine (BMS-212702)
M11	10	491	**MS^2:** 473, 315, 297. **MS^3 on 315:** 297, 281, 280, 159.	Glucuronide of monohydroxylated *O*,*N*-didesmethyl brasofensine[(c)]
M12	4	473	**MS^2**: 197	Glucuronide conjugate of a phase I metabolite
M13	14	505	**MS^2**: 455, 329, 311, 297. **MS^3 on 329**: 297, 281, 271, 238.	Glucuronide of monohydroxylated N-desmethyl brasofensine[(c)]
M14	6	491	**MS^2:** 315, 297, 270. **MS^3 on 297:** 279, 270, 252.	Glucuronide of monohydroxylated *O*,*N*-didesmethyl brasofensine[(c)]

M15	20	491	**MS^2:** 315, 297, 270. **MS^3 on 297:** 279, 270, 264, 252.	Glucuronide of monohydroxylated *O*,*N*-didesmethyl brasofensine[(c)]
M16	5	473	**MS^2**: 197	Glucuronide conjugate of a phase I metabolite
M17	9	505	**MS^2**: 329, 311, 297. **MS^3 on 329**: 311, 297, 281, 271.	Glucuronide of monohydroxylated *N*-desmethyl brasofensine[(c)]
Total	88[(b)]			

(a) Trace: Trace amount of the metabolite that was detected by LC/MS but not by radioflow-detector.

(b) The remaining urinary radioactivity was distributed among several unidentified peaks, none of which individually accounted for more than 5% of the total urinary radioactivity.

(c) This metabolite could be either the E-isomer or its Z-isomer.

(d) Major or significant product ions.

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Figure 1

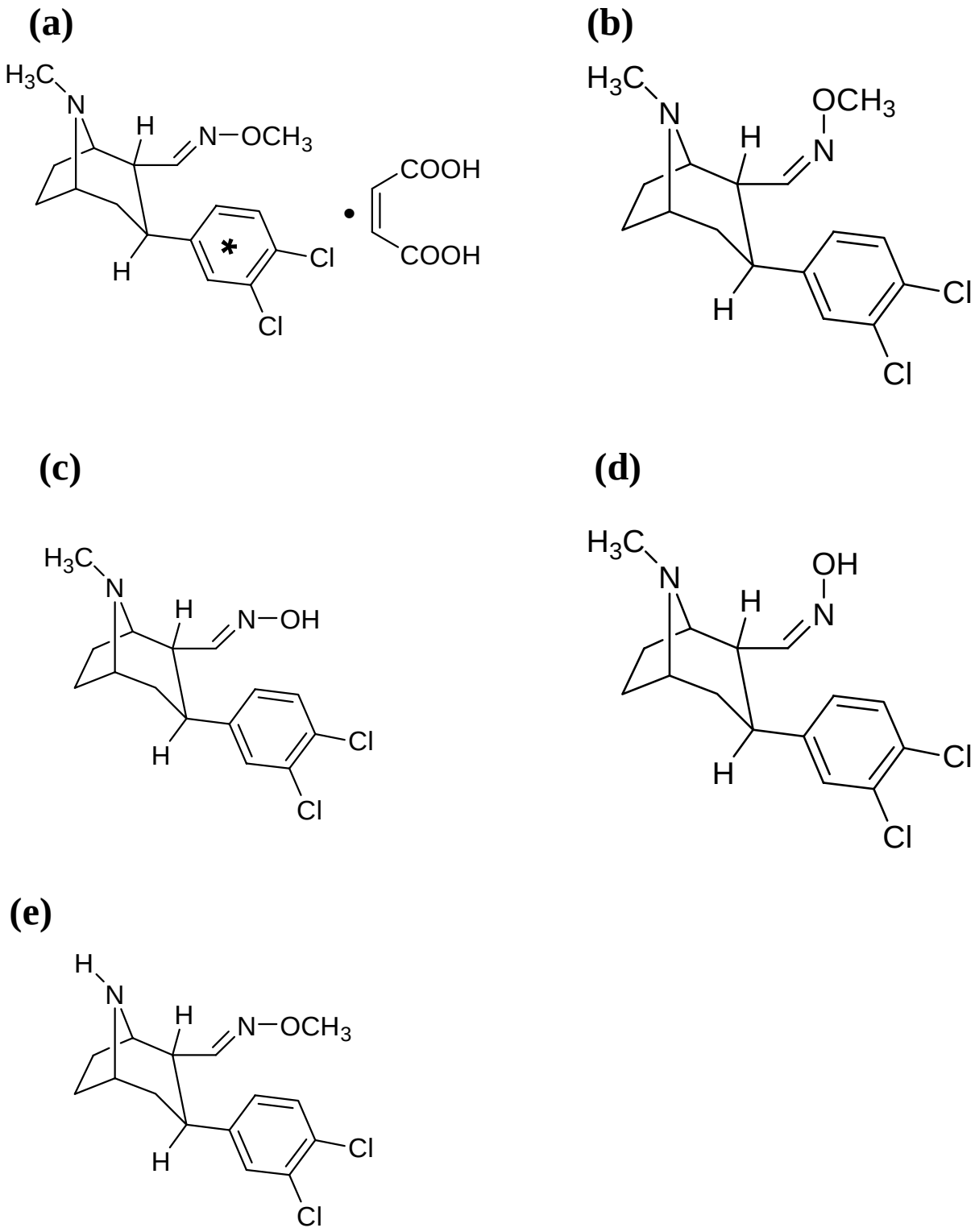

Figure 2

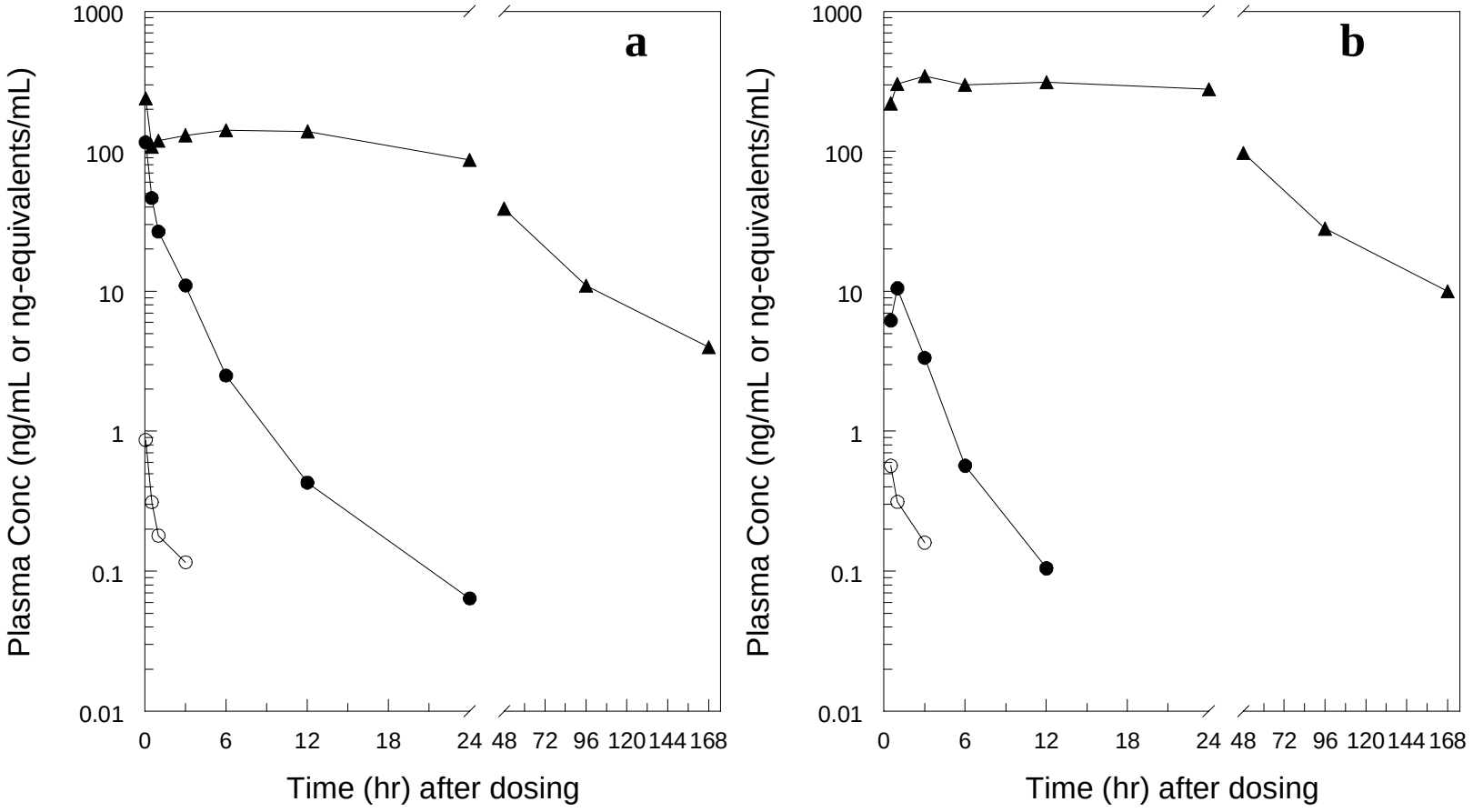

Figure 3

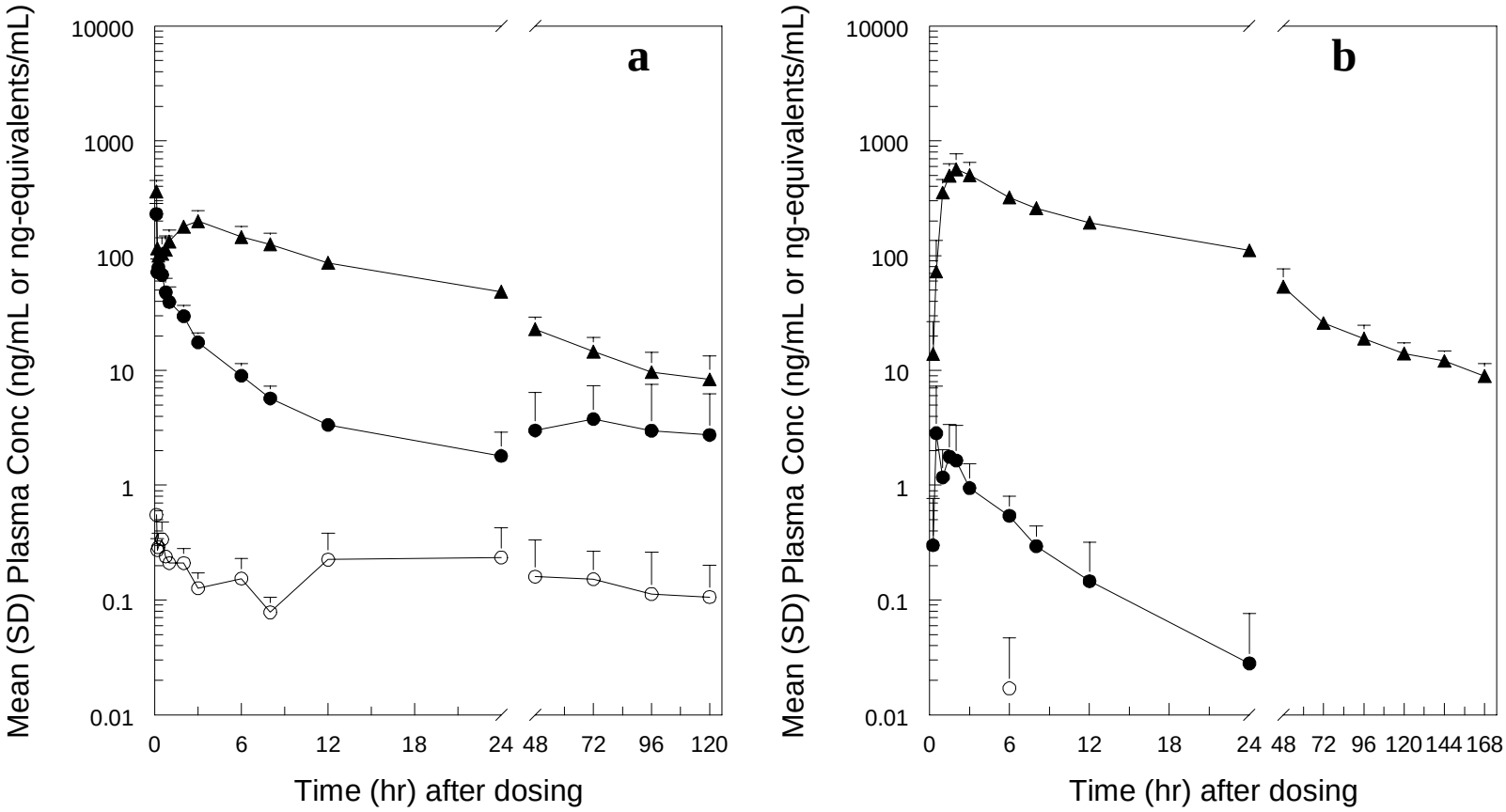

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Figure 4

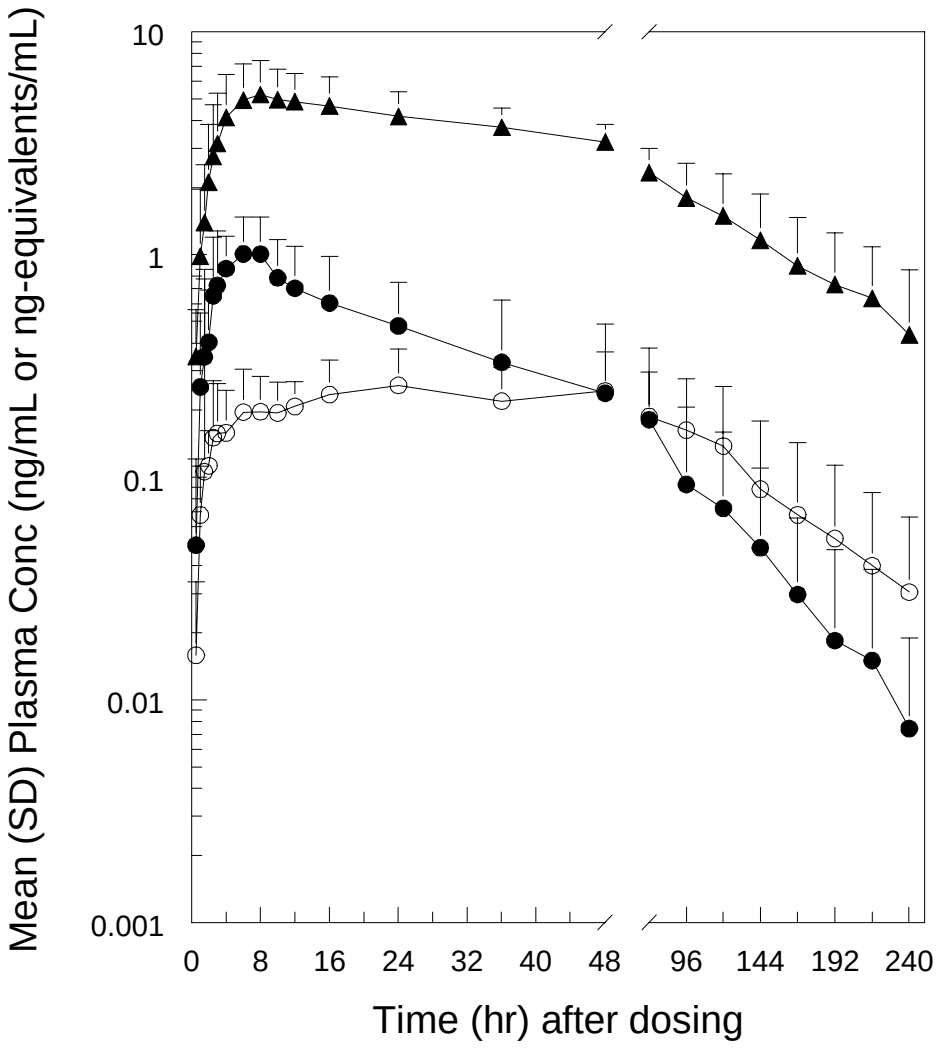

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Figure 5

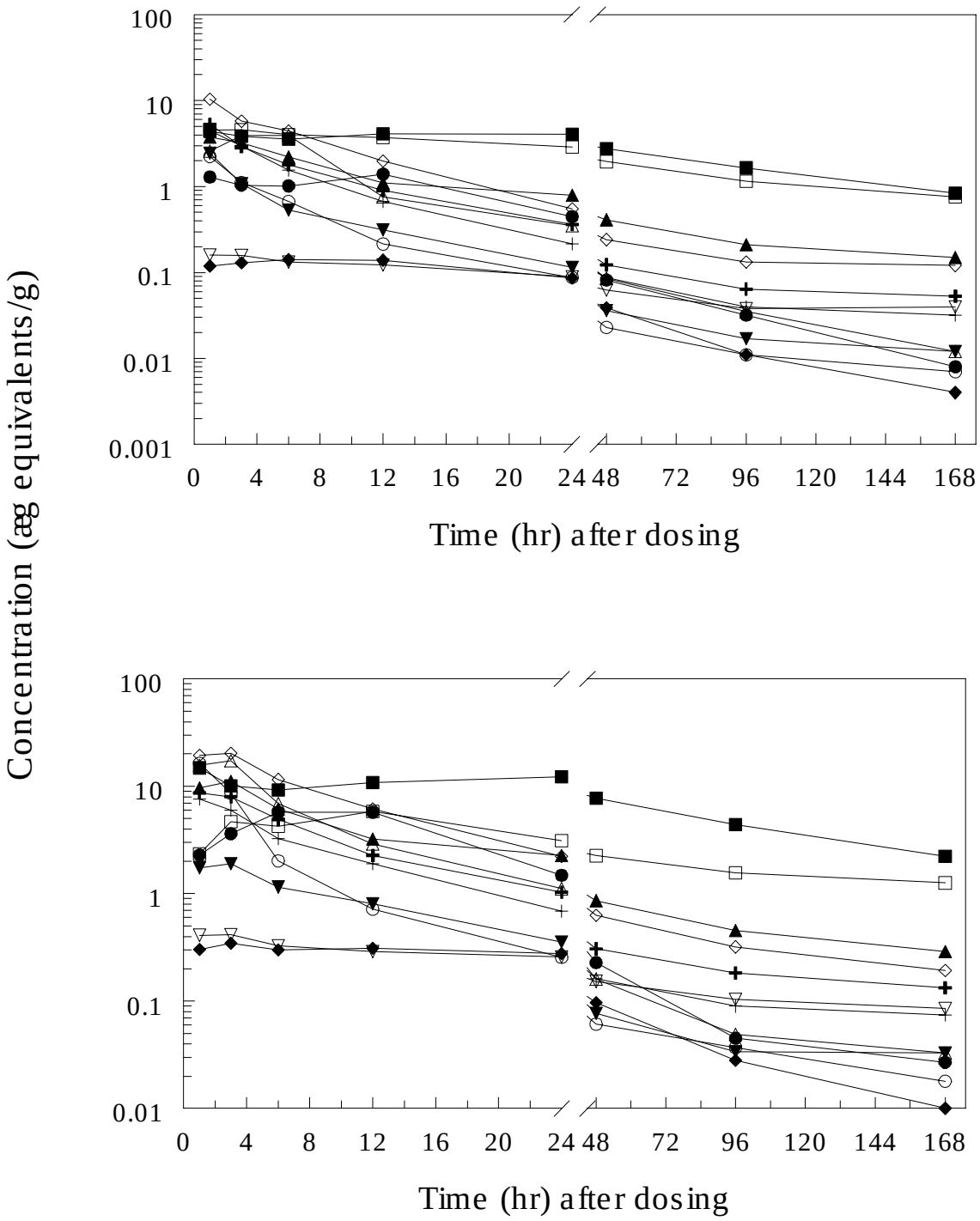

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Fig. 6

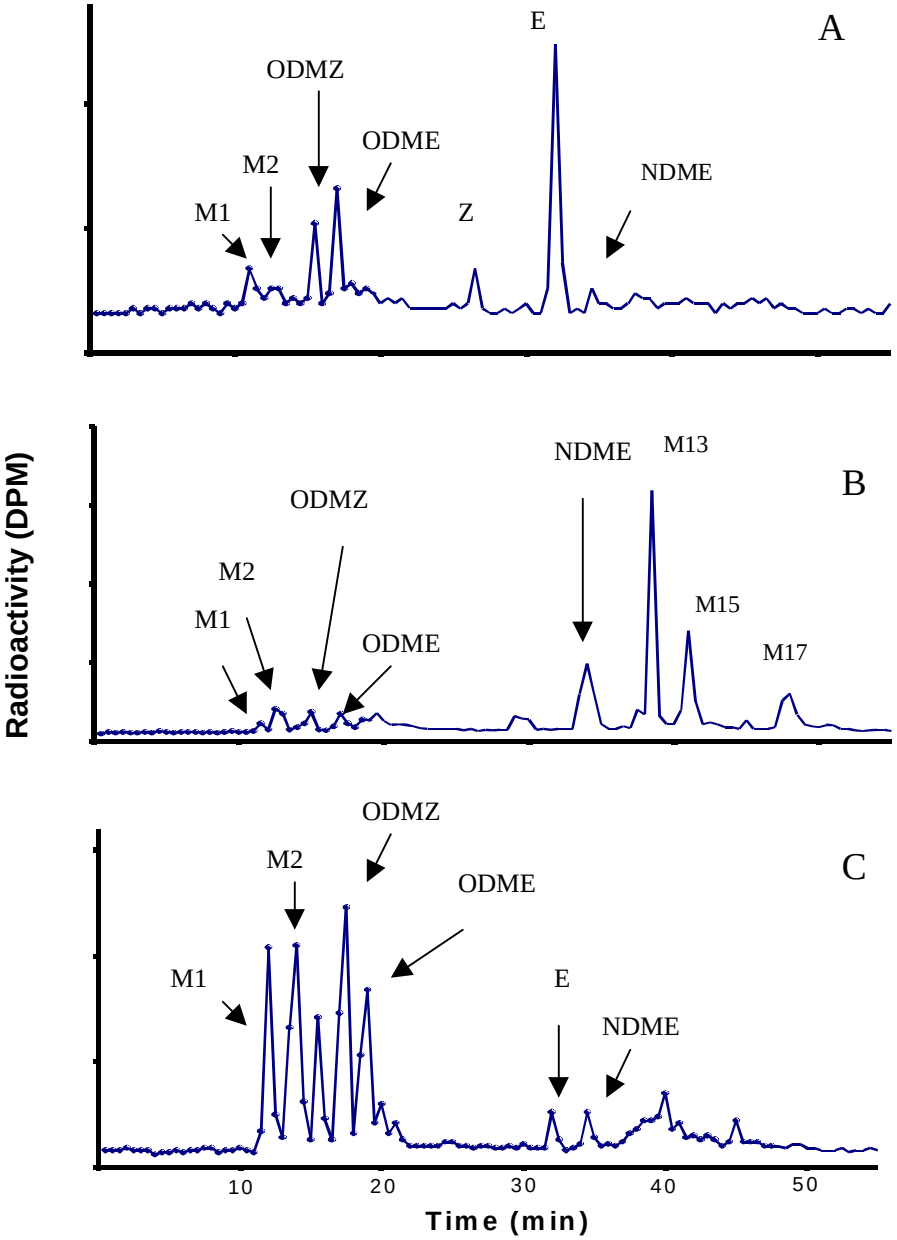

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Figure 7.

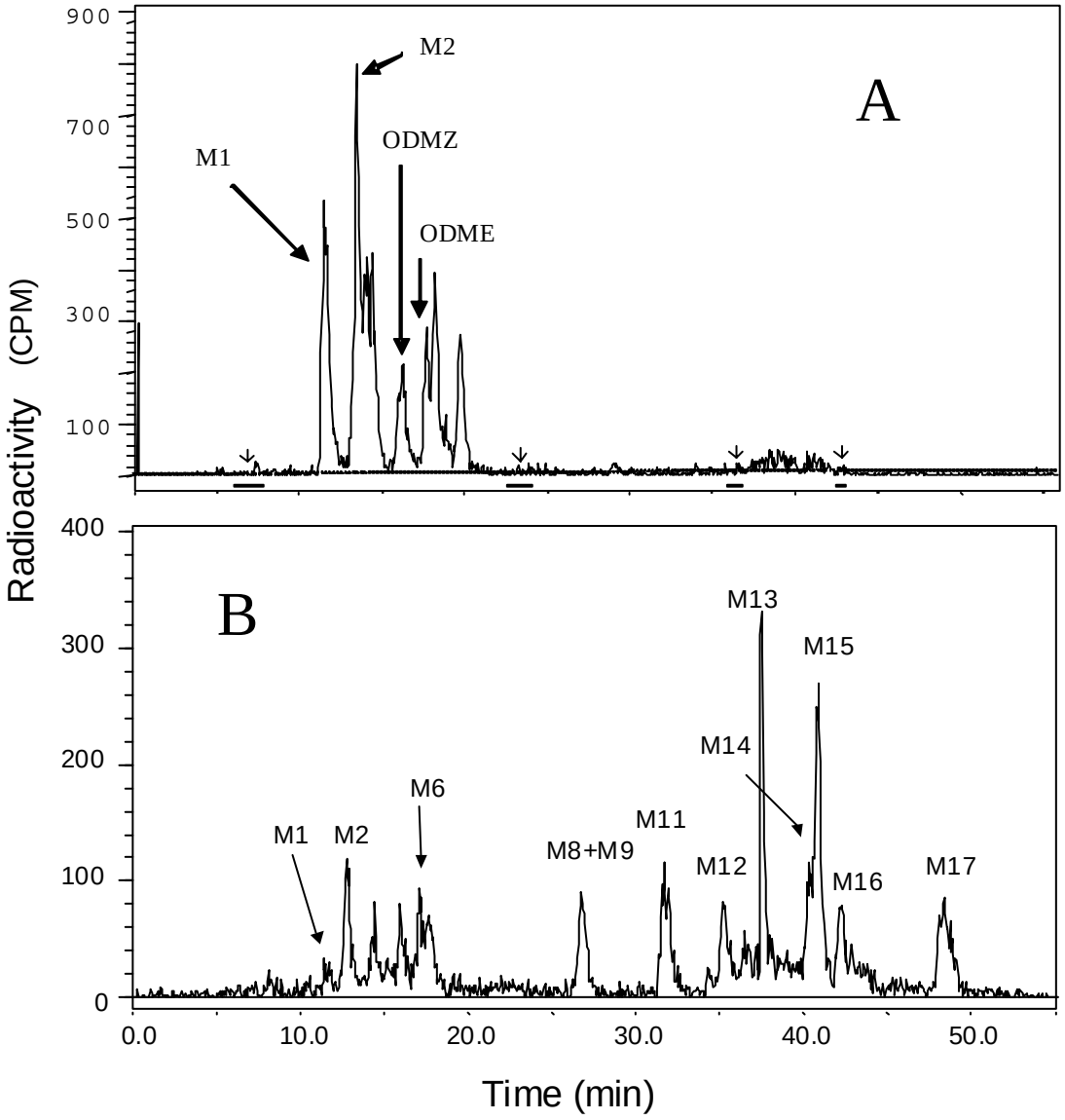

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Figure8

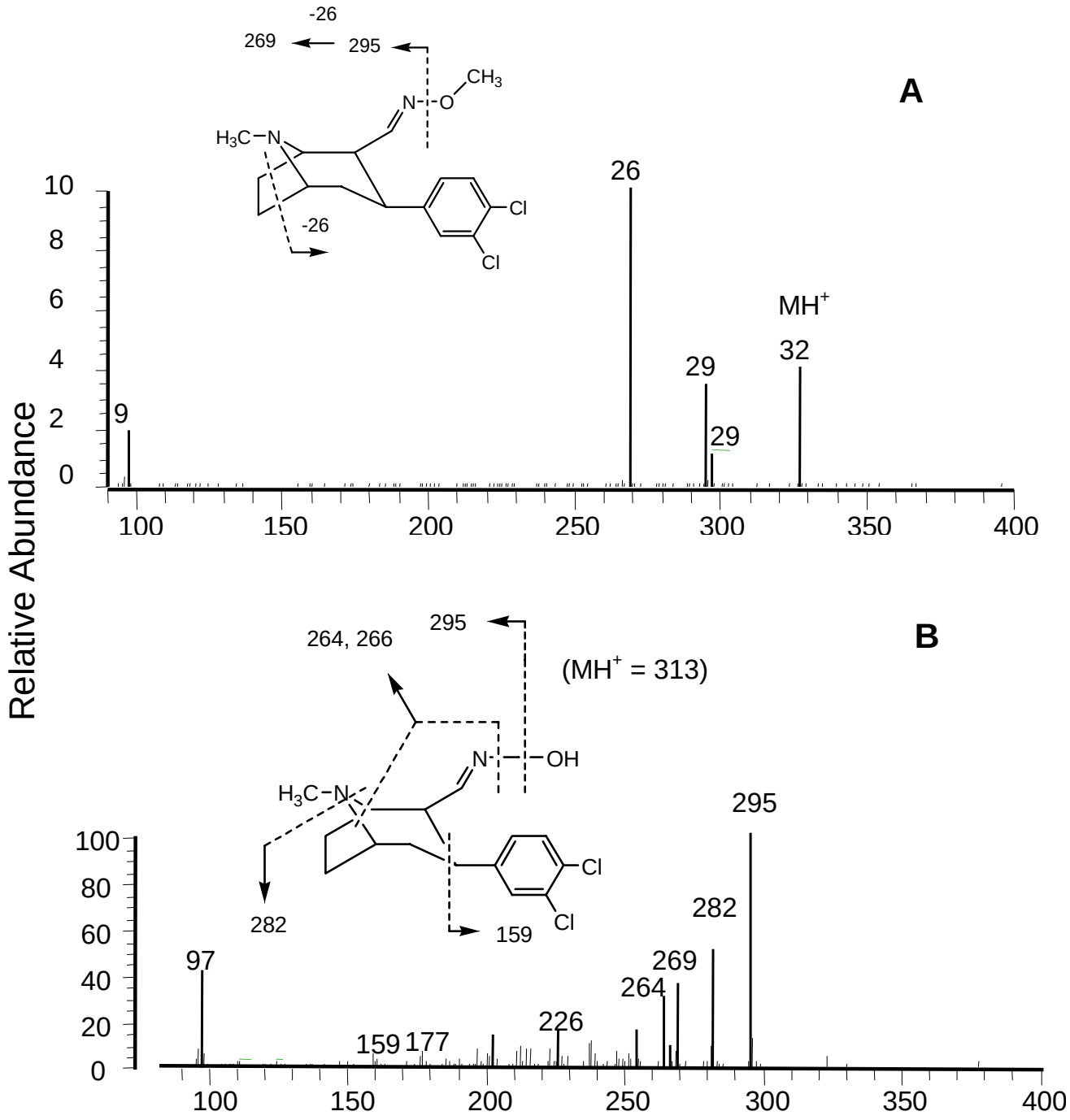

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Figure 9

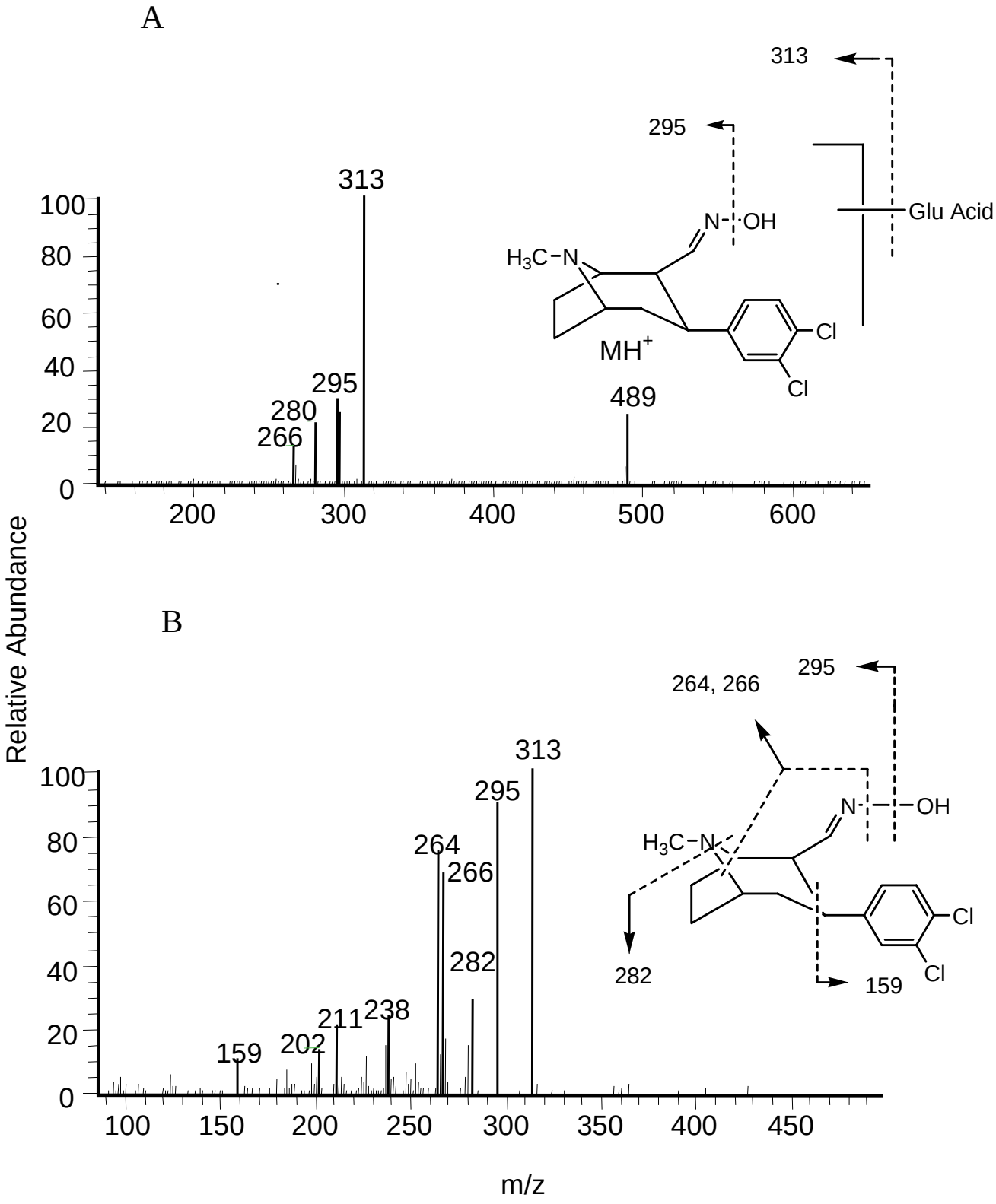

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Figure 10

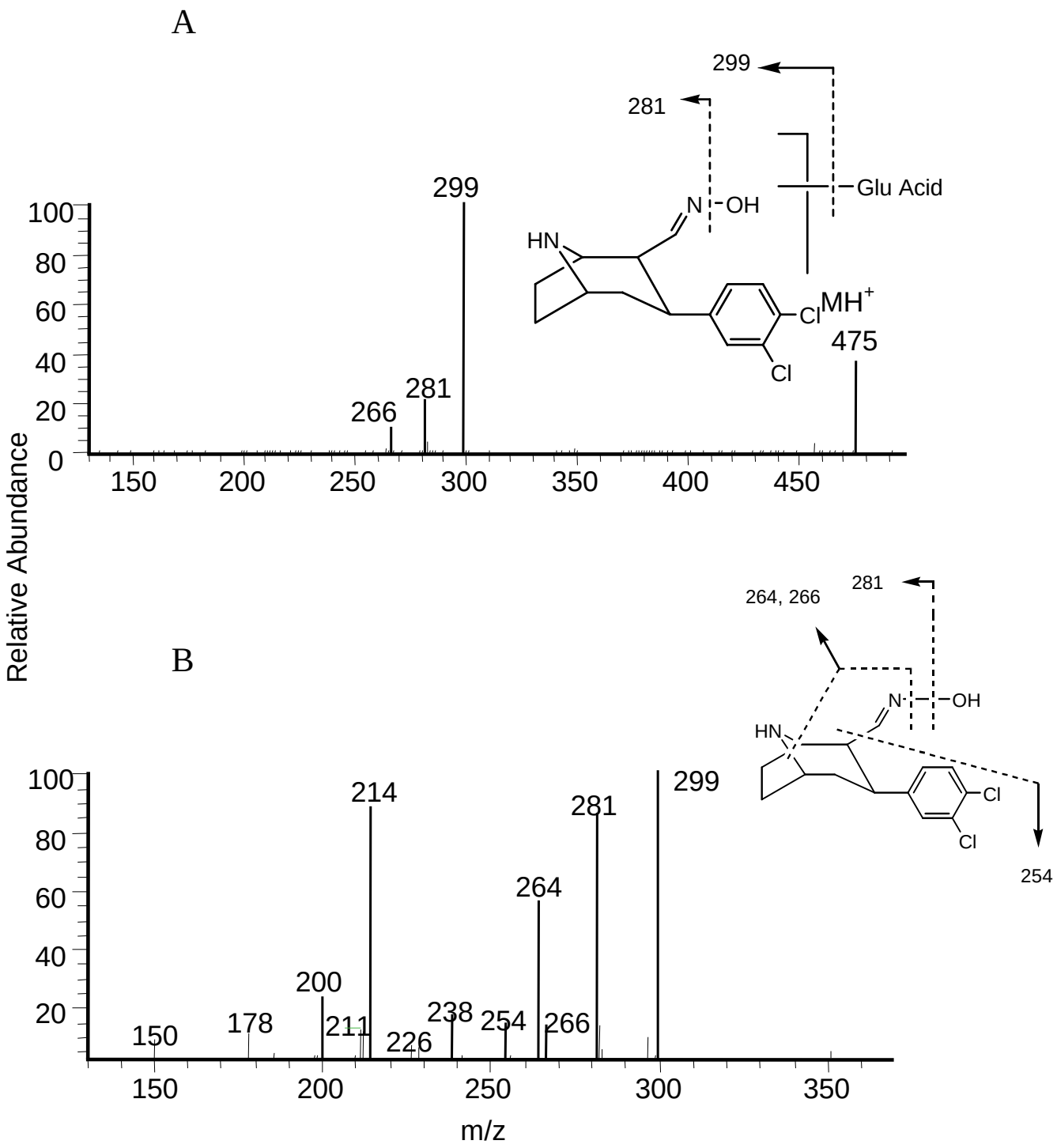

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Figure 11.

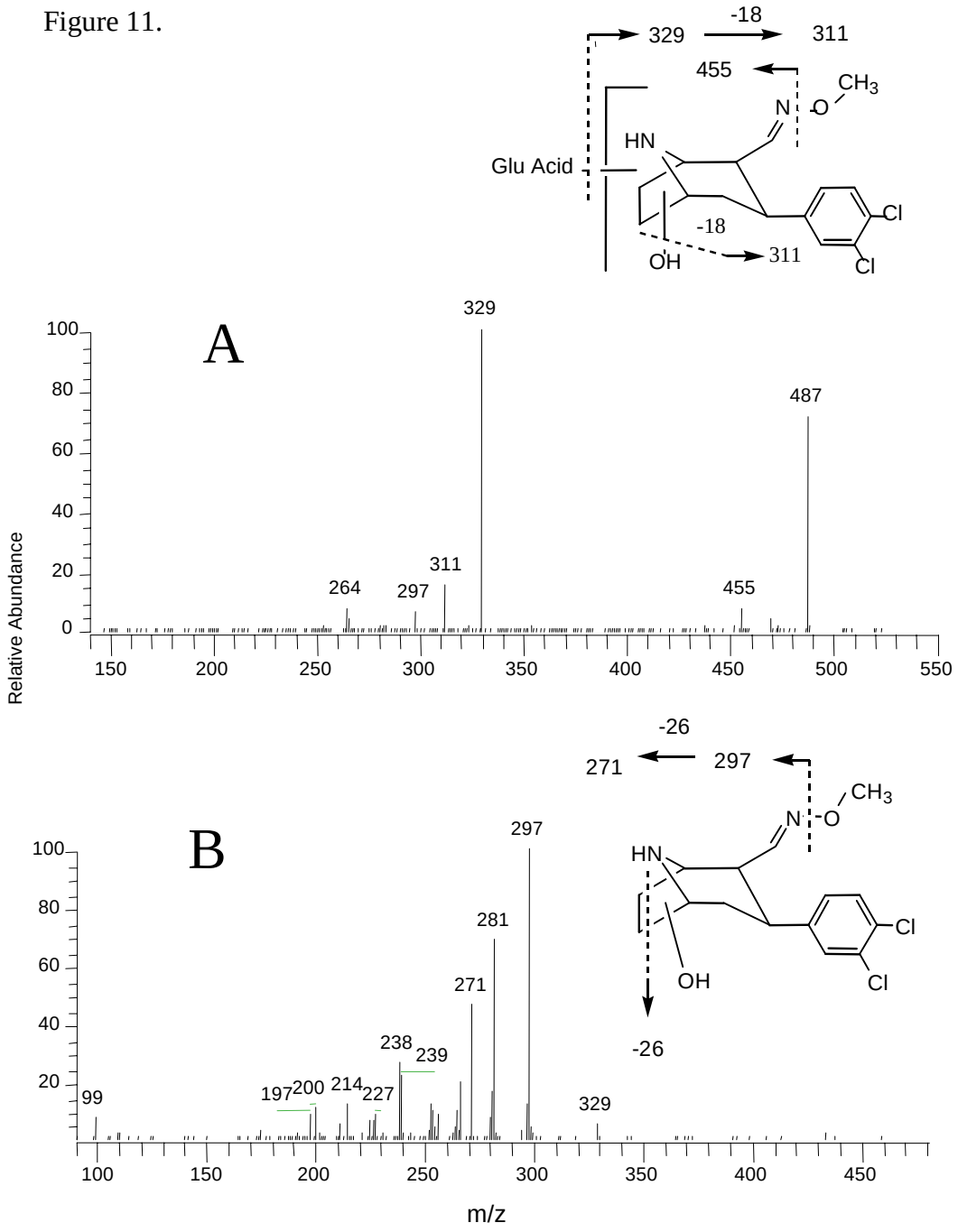

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Figure. 12.

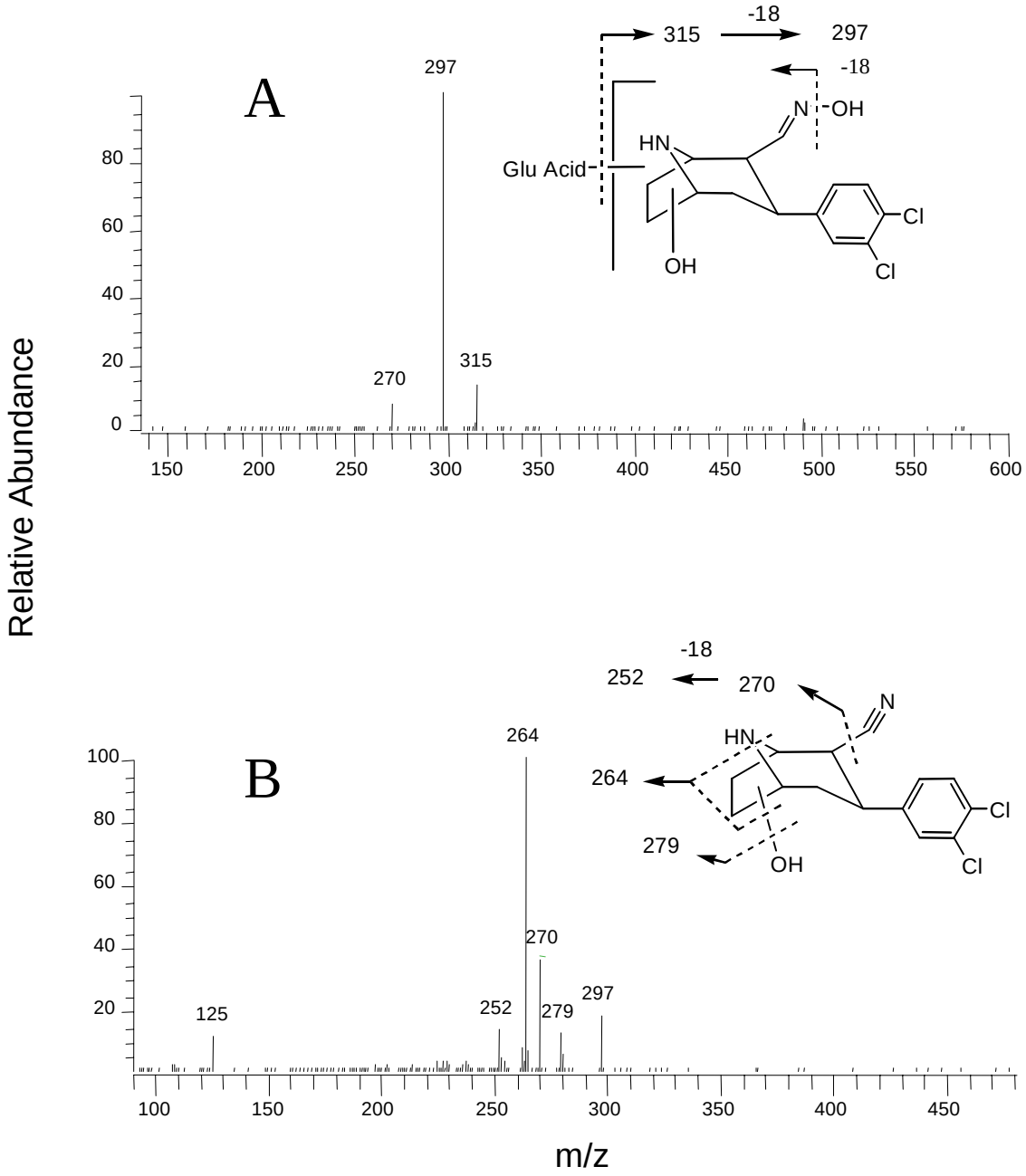

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Figure 13