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METABOLISM AND DISPOSITION OF VARENICLINE, A SELECTIVE α4β2 ACETYLCHOLINE RECEPTOR PARTIAL AGONIST, IN VIVO AND IN VITRO

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Abbreviations: UGT: uridine glucuronyl transferase

ABSTRACT

The metabolism and disposition of varenicline, a partial agonist of the nicotinic acetylcholine receptor for the treatment of tobacco addiction, was examined in rats, mice, monkeys, and humans after oral administration of [^{14}C]varenicline. In circulation of all species, the majority of drug-related material was comprised of unchanged varenicline. In all four species, drug-related material was primarily excreted in the urine. A large percentage was excreted as unchanged parent drug (90, 84, 75, and 81% of dose in mouse, rat, monkey, and human, respectively). Metabolites observed in excreta arose via N-carbamoyl glucuronidation, and oxidation. These metabolites were also observed in the circulation, in addition to metabolites that arose via N-formylation and formation of a novel hexose conjugate. Experiments were conducted using in vitro systems in order to gain an understanding of the enzymes involved in the formation of the N-carbamoylglucuronide metabolite in humans. N-Carbamoyl glucuronidation was catalyzed by UGT2B7 in human liver microsomes when incubations were conducted under a CO_2 atmosphere. The straightforward dispositional profile of varenicline should simplify its use in the clinic as an aid in smoking cessation.

Smoking is the leading preventable risk to human health. In 2000 it was estimated that there were 1.25 billion smokers worldwide and that nearly 5 million premature deaths globally were attributable to smoking (Taylor and Bettcher, 2000; Ezzati and Lopez, 2003), and on average chronic smokers die ten years earlier than nonsmokers (Doll, et al., 2005). By 2030, the number of smokers is estimated to grow up to more than 1.6 billion, with the number of associated annual deaths estimated to reach 10 million (Mackay and Eriksen, 2002). Tobacco users become physically dependent on nicotine, making it difficult for them to stop using tobacco products. To date, drug therapeutic options designed to assist tobacco users quit include nicotine replacement therapies (e.g. nicotine gum, nicotine dermal patches, etc) and bupropion. Psychological therapies are also used for smoking cessation, such as smoking cessation programs. However, efficacy for these therapies is limited, and several shortcomings exist, warranting development of a better treatment option. Even when tobacco users are able to cease using these products for periods of time, it is common for them to reinitiate use habits several months later.

Varenicline (7,8,9,10-Tetrahydro-6,10-methano-6H-pyrazino[2,3-h][3] benzazepine; Figure 1) is a partial agonist of the nicotinic $\alpha 4\beta 2$ acetylcholine receptor, with demonstrated efficacy as a smoking cessation agent (Coe, et al., 2005). Varenicline is active in animal models of nicotine dependence. To gain an understanding of the efficacy and safety of new drugs, the total disposition and metabolism of the agent must be determined. Metabolites that are present in humans and animal species used in assessment of pharmacological action need to be identified in order to determine whether they could contribute to desired effect. Also, metabolites present in humans need to be

considered in risk assessments made from animal safety studies, to ensure that metabolites present in humans are also present in animal species used to determine potential toxicity.

Varenicline is a simple, small organic molecule, providing limited structural entities for biotransformation reactions. In fact, a large percentage of the administered dose of varenicline is excreted unchanged. However, while there is little metabolism of this compound, some of the metabolites that were observed (vide infra) were unusual. The objective of this study was to determine the routes of excretion of varenicline and metabolites in humans and animal species, and to elucidate the structures of varenicline metabolites.

MATERIALS AND METHODS

Materials. Varenicline succinate and N-formylvarenicline were obtained from the Process Research and Development Department, Pfizer, Groton, CT. UDPGA, alamethicin, and D-saccharic acid 1,4-lactone were obtained from Sigma-Aldrich (St. Louis, MO). Human liver microsomes pooled from 53 individual donors and recombinant heterologously expressed human UGT enzymes were purchased from BD-Gentest (Woburn, MA). Synthetic procedures for the preparation of radiolabelled varenicline and 2-hydroxyvarenicline are available in the on-line Supplemental Information of this journal.

Dosing of Animals and Collection of Samples. Preclinical species were administered a single oral dose of [^{14}C]varenicline by gavage of aqueous solutions. The following dose levels, specific activities, and solution volumes were used: Sprague-Dawley rat 3.0 mg/kg, 25 μCi/250 gm rat, 2 mL/kg; CD-1 mouse:3.0 mg/kg, 20 μCi/20 gm mouse, 10 mL/kg; cynomolgus monkey: 0.08 mg/kg, 200 μCi/4 kg monkey, 2 mL/kg.

Rats were divided into four groups. In group 1 (N = 3/sex) animals were dosed and housed in stainless steel metabolism cages for collection of excreta and determination of mass balance. Urine samples were collected in intervals of 0-8 hr, 8-24 hr, followed by daily collection for one week. Fecal samples were collected daily for one week. These samples were used in the determination of mass balance, excretory mechanisms of varenicline drug-related material, and identification of metabolites. In group 2 (N = 2/sex), cannulae were surgically implanted into the bile duct under anaesthesia, and the rats were allowed to recover overnight. Bile samples were collected from these animals over a 48 hr post-dose period. These samples were used in the

identification of metabolites in bile. In group 3 (N = 3/sex), cannulae were surgically implanted into the jugular vein under anaesthesia followed by a one day recovery period. Blood samples (0.7 mL) were collected serially from each animal at 0.5, 1, 2, 4, 8, and 24 hr post-dose and processed to obtain serum, with 2 mL of blood from donor animals used to replenish blood volume in the study animals. These samples were used in the determination of pharmacokinetics of varenicline and total drug-related radioactivity. In group 4 (N = 12/sex), animals were euthanized with CO_2 followed by collection of blood at 1, 2, 4, and 8 hr post-dose. These samples were used in the identification of circulating metabolites.

Mice were divided into two groups. In group 1 (N = 9/sex), mice were housed in three groups of three/sex in metabolism cages for collection of excreta. Urine and feces were collected in 24 hr intervals for eight days. These samples were used in the determination of mass balance, excretory mechanisms of varenicline drug-related material, and identification of metabolites. In group 2 (N = 18/sex; N = 6/sex/sampling time), animals were terminated for collection of blood samples at times of 1, 4, and 12 hr post-dose. These samples were used in the quantitation of varenicline and total radioactivity and in the identification of circulating metabolites.

Monkeys were divided into two groups. In group 1 (N = 2/sex), monkeys were housed in metabolism cages for collection of excreta and determination of mass balance. Urine samples were collected in intervals of 0-8 hr, 8-24 hr, followed by daily collection for ten days. Fecal samples were collected daily for ten days. Blood samples (3.0 mL) were collected from the saphenous vein at 0.5, 1, 2, 4, 8, 12, 24, 48, and 72 hr post-dose. Blood was processed to obtain plasma. These samples were used in the quantitation of

varenicline and total radioactivity as well as the identification of circulating metabolites. In a second group (N = 1/sex) animals possessed a chronic indwelling catheter in the bile duct. In these animals, bile was collected for two days post-dose, with bile salt replacement therapy throughout the collection period.

Dosing of Human Volunteers and Collection of Samples. Six healthy human male subjects were administered 1.0 mg/100 μCi [^{14}C]varenicline in water (240 mL). A dose of 1.0 mg was selected because this is an efficacious dose. Three were non-smoker subjects and three were smokers. Blood samples (sufficient to provide 6 mL serum) were collected for pharmacokinetic evaluation of varenicline and total radioactivity at time points of 0 (just prior to dosing), 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120, 144, 168, and 192 hr post-dose. Additional blood sufficient to provide a minimum of 20 mL serum were collected at 1, 4, 8, 12, and 24 hr post-dose for profiling and identification of metabolites. All blood samples were processed to obtain serum and stored frozen prior to analysis. Urine samples were collected prior to dosing and at intervals of 0-12, 12-24, and daily thereafter for one week. Feces were collected in daily intervals through four days post-dose. Samples were stored frozen until the day of analysis.

In Vitro Incubations. The N-carbaoylglucuronidation of varenicline was studied in vitro under the following conditions. Pooled human liver microsomes or individual human recombinant UGT enzymes were first treated with alamethicin, $MgCl_2$, and sodium bicarbonate (pH adjusted to 7.5) in a volume of 0.20 or 0.32 mL on ice for 15 min. This was followed by addition of varenicline and saccharolactone and warming to 37^{o}C over 5 min under CO_2. Incubations were commenced by addition of UDPGA in a final

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incubation volume of 0.4 ml. Final assay concentrations were: microsomes (0.5 mg/ml), alamethacin (0.05 mg/ml), $NaHCO_3$ (100 mM), $MgCl_2$ (10 mM), saccharolactone (5 mM), varenicline (20-2000 μM), and UDPGA (5 mM). Incubations were conducted in a Lucite container submerged in a heated water bath that permitted the continuous flow of CO_2. The CO_2 was passed through a gas warmer to prevent cooling of the incubations. After 2 hr, incubations were terminated by the addition of 0.8 ml CH_3CN. The incubation time and protein concentration used were determined to be linear in initial experiments.

Samples were spun in a centrifuge (1850 x g) at ambient temperature to remove precipitated protein and the supernatant was evaporated under N_2 at 35°C. The residue was reconsitituted in 0.1 ml HPLC mobile phase and injected (0.05 ml) onto a Monitor C18 column (150 x 4.6 mm; 5μ) equilibrated in 40% methanol in 20 mM acetic acid adjusted to pH 4 with NH_4OH at a flow rate of 0.8 ml/min. The elution conditions were isocratic. The effluent was introduced into the ionspray source of a Sciex API3000 mass spectrometer (see below). Varenicline N-carbamoylglucuronide was monitored by selected reaction monitoring of m/z 432 → 256 and eluted at 5.6 min. Quantitation was done using a standard curve of varenicline N-carbamoylglucuronide ranging from 2.5–1000 ng/ml. An authentic standard of varenicline N-carbamoylglucuronide for use in quantitation was not able to be prepared. Therefore, an approach was employed such that a "standard solution" of this metabolite was prepared by administering a dose of [^{14}C]varenicline of known specific activity (6.5 mCi/mmol) to a rat and collecting bile for 28 hr. The concentration of total drug-related material in bile was determined followed by profiling by radiometric HLPC. This yielded a standard solution of

varenicline N-carbamoylglucuronide of 3.03 μg/ml, which was diluted into control matrix to generate the standard curve. Mass spectral response was monitored for the standards, and correction was made for the ratio of [^{12}C]/[^{14}C]. This type of approach has been previously described (Dow, et al., 1994)

Quantitative Analysis of Varenicline in Serum or Plasma Samples. Circulating varenicline concentrations were measured using a method involving liquid extraction and HPLC/MS/MS analysis. To plasma or serum samples (0.1 mL) was added CP-533,633, a structural analogue of varenicline (50 μL of 0.25 μg/mL in methanol), as an internal standard and 1N sodium hydroxide (0.5 mL). The alkalinized samples were subjected to liquid extraction with methyl t-butyl ether (3 mL). The aqueous layer was frozen in a dry ice-acetone bath and the organic fraction was decanted and evaporated under N_2. The residue was reconstituted in 100 μL HPLC mobile phase.

Reconstituted extracts were injected (50 μL; CTC Analytics Leap Autoinjector, Carrboro, NC) onto a C18 Monitor column (4.6 x 150 mm; 5 μ Column Engineering, Ontario, CA) equilibrated in 10 mM NH_4OAc (pH unadjusted)/CH_3OH (25:75) at a flow rate of 0.8 mL/min (Shimadzu LC10AD Pumps, Columbia, MD). The effluent was split 1:1 and was introduced into a Turbo Ionspray™ source of a Sciex API 3000 mass spectrometer (Applied Biosystems/SCIEX, Thornhill, Ontario, Canada) operated in the positive ion mode. The temperature of the probe was 400°C, the orifice voltage was 55V, the collision energy was 30V, and the collision gas setting was 10. Varenicline and internal standard were monitored by molecular reaction monitoring of 212.2→169.1 for the parent, 216.2→173.1 for the radiolabeled parent and 240.2→197.1 for the internal

standard. Varenicline and internal standard eluted at 2.5 and 3.0 min, respectively. The dynamic range of the assay was from 0.5 to 100 ng/mL.

Determination of Radioactivity and Processing of Samples for Profiling and Metabolite Identification by HPLC-MS/MS. Aliquots of urine and bile were counted in triplicate by liquid scintillation counting; plasma or serum samples were counted in singlicate; fecal samples were homogenized with water or water/acetonitrile mixtures, aliquots were permitted to air dry followed by combustion on a Packard Oximate 80 (Packard, Downers Grove, IL).

For metabolite profiling by HPLC-MS/MS, urine samples from each animal were pooled such that daily samples containing a total of at least 80% of the radioactivity excreted in urine were combined. Samples were spun in a centrifuge (1850 x g) at ambient temperature to remove particulate materials, and supernatants were injected directly on HPLC. Bile samples from each animal were pooled, spun in a centrifuge (1850 x g) at ambient temperature to remove particulate materials, and supernatants were injected directly on HPLC. Fecal homogenates were pooled such that daily samples containing a total of at least 80% of the radioactivity excreted in feces were combined. (Since so little radioactivity was excreted in feces in human, these samples were not analyzed for metabolite profile.) Fecal homogenate samples were treated with methanol containing 1% acetic acid for 4 days to extract radioactivity. Extraction recovery using this method ranged from 83 to 99%. Pooling of rat plasma samples was done by combining equal aliquots from each animal/sex for a given sampling time. For the other species, samples were combined for each individual according to the method of Hamilton et al. (1981), such that each sample was representative of total exposures

(AUC) to metabolites relative to each other. Pooled samples were treated with CH_3CN (4:1) to precipitate plasma proteins, the samples spun in a centrifuge (1850 x g) at ambient temperature, the supernatant transferred to a new tube, the pellet was resuspended in water (1 mL), and washed with CH_3CN (4 mL). The supernatants were combined, and the solvent removed by evaporation under N_2. The extraction procedure yielded recoveries of total radioactivity of at least 95% in all species.

Profiling and Identification of Metabolites by HPLC-MS/MS. The same HPLC/MS equipment used in the quantitation of varenicline was used for metabolite profiling. The mobile phase conditions were altered to effect chromatographic separation of metabolites. The mobile phase consisted of 10 mM NH_4OAc (pH unadjusted) (Solvent A) and methanol (Solvent B) at a flow rate of 1.0 mL/min. A gradient was applied consisting of 95% A held for 8 min, followed by a linear gradient to 60% B over the next 30 min. The column was washed and reequilibrated after each injection. The effluent was split (1:9) between the mass spectrometer and a radiometric flow detector (Beta-RAM, Inus Systems, Tampa, FL) containing a 0.25 mL Li solid radioactivity detection cell.

HPLC-MS-NMR. The LC/MS-NMR system consisted of Agilent1100 binary pump (Agilent Technologies, CA.), a Bruker Bio-Spin BSFU-0 column oven (Bruker Bio-Spin, MA), interfaced with a Bruker Bio-Spin photodiode array detector, a Bruker Bio-Spin BNMI interface using 20:1 split, a Bruker Daltonics Esquire 3000 ion trap MS (Bruker Daltonics, MA.) equipped with an electropsray source, a Bruker Bio-Spin BPSU-36 peak storage unit, and a Bruker BioSpin 500MHz Avance DRX Spectrometer equipped with a 4mm ^{1}H-^{13}C inverse z-gradient LC flow probe. Chromatographic separation was

achieved on a Curosil PFP 3µm 3X150mm (Phenomenex, Torrance, CA) at ambient temperature. A gradient mobile phase of (0.1% TFA-d in D_2O)-(acetonitrile-d_3) 100-0 to 70-30 over 60 minutes at a flow rate of 0.5mL/min was used to achieve chromatographic separation. Five percent of the effluent was split off post-column and diluted with 9:1 acetonitrile:D_2O containing 0.1% acetic acid-d_4 at a flow rate of 250µL/min prior to entering the MS. The remaining 95% was monitored by UV at 235nm. The metabolite peak at 18.83 minutes (m/z=231; $[M+D]^+$) and the parent peak at 20.23 (m/z=214; $[M+D]^+$) were stored in the BPSU-36 unit using the loop storage technique and subsequently introduced into the NMR spectrometer. ^{1}H NMR and gCOSY spectra were obtained at 298 K on both peaks using double presaturation of solvent NMR resonances. All chemical shifts are reported in ppm downfield from TMS as referenced from the shift of residual protons in acetonitrile-d_3 at 1.94 ppm.

Calculations. Pharmacokinetic parameters were calculated by a noncompartmental model using WinNonLin v2.1 (Pharsight, Cary, NC). AUC(0-t_{last}) was calculated using the linear trapezoid method. The elimination rate constant (k_{el}) was determined by linear regression of the log concentration vs. time data during the last observable elimination phase. Half-life was calculated as $0.693/k_{el}$. AUC(0-inf) was calculated as the sum of AUC(0-t_{last}) and AUC(t_{last}-inf). The latter was determined by dividing the concentration estimated at t_{last} from the aforementioned regression by k_{el}. C_{max} and T_{max} were taken directly from the concentration vs. time data. Enzyme kinetic parameters were calculated using the Enzyme Kinetics module of SigmaPlot v8 (SPSS, Chicago, IL).

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RESULTS

Excretion of [^{14}C]Varenicline Drug-Related Material in Animals and Humans. After oral administration of varenicline, excreta were collected and analyzed for radioactivity, a measure of the total drug-related material. Mean total recovery was high, with greater than 87.5% of the dose recovered (Table 1). In human, almost all of the recovered dose was excreted in urine (99% of recovered material), while in the animal species up to 25% of recovered material was in the feces. In mouse and rat, a greater percentage of radioactivity was excreted early compared to monkey and human (Figure 2). This is consistent with the short half-lives observed in the pharmacokinetic evaluation.

Pharmacokinetics of Varenicline and Total Radioactivity in Animals and Humans. Pharmacokinetic data for varenicline and total drug-related material are listed in Table 2. The half-lives for both parent drug and total radioactivity were substantially longer in monkey and human than in rat and mouse. Declines in circulating concentrations were first-order for parent and total radioactivity (Figure 3). Half-lives were the same, or slightly longer for total radioactivity vs parent drug, suggesting that metabolites have a slightly longer half-life. Unchanged varenicline constituted the majority of drug related material in circulation. Comparison of the varenicline and total radioactivity AUC values yields the result that parent comprises 70, 55, 63, and 79% of total drug-related material in mouse, rat, monkey, and human, respectively. A similar relationship was obtained for C_{max}.

Metabolite Profiles in Excreta of Animals and Humans. In urine from the four species, there was a total of six metabolites observed plus parent drug. Radiochromatograms of urine in each species are shown in Figure 4. Fecal homogenates contained only

unchanged parent drug, and bile samples from rat and monkey only contained the N-carbamoyl glucuronide conjugate (data not shown). Unchanged drug comprised a total of 90, 84, 75, and 81% of the dose in mouse, rat, monkey, and human, respectively (Table 3). Adjusting these values for the total recovery and assuming that material not recovered had the same pattern of drug-related material as was observed in excreta, excretion of unchanged varenicline represents 95, 93, 86, and 92% of the dose in mouse, rat, monkey, and human, respectively. In human, two excretory metabolites were observed, an N-carbamoyl glucuronide conjugate and a metabolite that had undergone hydroxylation on the quinoxaline ring.

Metabolite Profiles in Circulation of Animals and Humans. A total of nine metabolites were observed in the circulation of the four species examined (Table 4). Of the nine observed, four were observed in human, including an N-formyl conjugate, an N-carbamoyl glucuronide, and N-hexose conjugate, as well as an unidentified metabolite with a protonated parent ion mass-to-charge of 226. Parent drug comprised the great majority of drug related material. Radiochromatograms of circulating metabolites are shown in Figure 5.

Structure Elucidation of Metabolites. Varenicline was largely excreted as unchanged drug and represented the vast majority of drug related material in circulation, however some metabolites were observed. However, in several instances the amounts of metabolites were limited, precluding definitive structural assignment, owing to the small amount of metabolism occurring for varenicline and the low doses administered.

Mass Spectrum of Varenicline: The collision-induced dissociation mass spectrum for the parent compound (*m/z* 212) yielded fragments associated with loss of the 2^{o} amine

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nitrogen (Figure 6). Fragments at *m/z* 195, 183, and 169 are proposed to arise via loss of ammonia, methyl imine, and dimethyl imine as neutral species, respectively. The fragment ion at *m/z* 141 represents fragmentation of the quinoxaline ring, as this fragment ion was also present in the product ion mass spectrum of [$^{14}C_2$]varenicline, in which the positions of isotopic substitution are the 2- and 3-positions of the quinoxaline ring. The fragment ion at *m/z* 168 is not readily identifiable and may arise via a radical process.

Metabolites M1 and M2: Two minor metabolites were observed in urine and circulation of the animal species (Tables 3 and 4). Small quantities precluded obtaining enough spectral information for definitive structure assignment. Both have protonated molecular ions at *m/z* 244, indicating the addition of 32 mass units to the parent. This could be indicative of two hydroxylations or formation of a lactam followed by hydrolysis to the amino acid. The fragment ion at *m/z* 29 indicates the loss of methylamine (Figure 6). The fragment ion at *m/z* 198 is suggestive of the neutral loss of formic acid, favoring the possibility of an amino acid metabolite, which could further lose ammonia to *m/z* 181. Methylation of M1 and M2 in methanolic HCl yielded products with molecular ions 14 mass units greater (m/z 258) suggesting the amino acid structures. M1 and M2 are likely diastereomers, and could arise via hydrolysis of intermediate lactam metabolites (see metabolites M3c and M3d, below).

Metabolite M3b (2-Hydroxyvarenicline): Metabolite M3b was observed in human urine, representing excretion of 2.9% of the dose. It possessed a molecular ion of *m/z* 228, and a similar fragmentation pattern to the parent drug, excepting the shift of 16 mass units for the fragment ions. The metabolite demonstrated an identical HPLC retention time,

CID mass spectrum, and [^{1}H]NMR spectrum as an authentic standard (Figure 7). In the NMR spectrum, proton resonances in the alkyl region are are similar or only slightly shifted between varenicline and the metabolite, indicating that the position of hydroxylation is on one of the quinoxaline positions. Additionally, the two aromatic singlet resonances in the parent spectrum (8.83 and 8.03 ppm; 2H each) have shifted to three singlet resonances (8.19, 7.79 and 7.38 ppm; 1H each) in the metabolite spectrum. Hence, the hydroxylation must have occurred on the aromatic moiety resulting in three non-equivalent proton resonances. Since there is no coupling between any of these protons, the hydroxylation must have occurred next to the nitrogen. This observation is confirmed by the gCOSY spectrum of the metabolite (data not shown); there are no correlation peaks for the aromatic resonances. While this metabolite was not observed in any of the three animal species using radiometric detection after administration of radiolabelled material, it was measured in the urine of rats using a specific HPLC/MS-MS method and shown to represent approximately 0.5% of a 3.0 mg/kg dose (data not shown).

Metabolites M3c and M3d: Two metabolites possessing protonated molecular ions of *m/z* 226 were observed in the circulation of mice, monkeys, and humans. This represents an addition of 14 mass units, suggestive of the addition of one oxygen and removal of two hydrogens. Abundance in circulation was too low to yield adequate spectral data; this was obtained by generating these two metabolites by incubating varenicline with monkey hepatocytes. The CID spectrum for metabolite M3c is shown in Figure 6. Loss of 45 mass units (*m/z* 181) is consistent with a neutral loss of formamide. Metabolites M3c and M3d showed similar ions in their CID spectra, but with different abundances.

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They are likely the diastereomeric lactams with the oxygen on either side of the nitrogen, and hence precursors of the carboxylic acids M1 and M2.

Metabolite M4 (Varenicline N-Carbamoylglucuronide): Metabolite M4, the N-carbamoyl glucuronide, was observed in rat, monkey, and human urine and circulation, and was also the only metabolite observed in rat and monkey bile. The CID mass spectrum of *m/z* 432 (addition of varenicline + CO_2 + glucuronide) yielded major fragment ions of *m/z* 256 (loss of glucuonide) and *m/z* 212 (parent), consistent with this structural assignment (Figure 6). Additionally, this metabolite was generated by incubation of liver microsomes and UDPGA in bicarbonate buffer under an atmosphere of CO_2. Such conditions have been previously applied in the generation of the N-carbamoyl glucuronide conjugate of carvedilol and sertraline (Schaefer, 1992; Obach, et al., 2005).

Metabolite M5 (N-Formylvarenicline): Metabolite M5 was identified as an N-formyl conjugate, and was present in the circulation of all four species examined. The molecular ion of *m/z* 240 represents an addition of 28 mass units to the parent compound. The fragment ions of *m/z* 212, 195, and 181 represent loss of C≡O, formamide, and N-methylformamide, respectively (Figure 6). The metabolite matched an authentic standard of this compound in HPLC retention time and CID spectrum.

Metabolite M7 (N-Glucosylvarenicline): Metabolite M7 was present in the circulation of rat, monkey, and human. The molecular ion was *m/z* 374, representing an addition of 162 mass units to the parent compound, and the CID spectrum yielded only *m/z* 356 as a product ion from *m/z* 374, along with other ions identical to those observed in the parent drug (Figure 6). Incubation of varenicline in a weakly alkaline solution of 1M glucose

yielded a peak with the same retention time and CID mass spectrum, suggesting that M7 is a glucose adduct of varenicline.

In Vitro Metabolism of Varenicline to N-Carbamoyl Glucuronide Conjugate. The metabolism of varenicline to varenicline N-carbamoylglucuronide was quantitatively studied in human-derived in vitro systems. In human liver microsomes, the enzyme kinetics were studied (Figure 8) up to a substrate concentration of 2 mM. The findings were such that an accurate determination of K_M could not be derived, since it is high (>1 mM). The capability of a panel of human recombinant UGT enzymes to catalyze varenicline N-carbamoylglucuronide was examined. Of the enzymes tested, only UGT2B7 demonstrated a capability to catalyze this reaction; the other UGT enzymes examined, UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B15, and 2B17 did not show formation of this metabolite above the lower limit of quantitation. The enzyme kinetics of varenicline N-carbamoylglucuronidation catalyzed by UGT2B7 are shown in Figure 8.

DISCUSSION

Varenicline is a promising new agent in the treatment of tobacco addiction (Coe, et al., 2005). As such, it is important to possess an understanding of the metabolism and disposition of this agent in humans as well as animal species that are used in safety assessments. Overall, the metabolic and excretory profile of varenicline is simple compared to most drugs (Figure 9). The majority of drug-related material is excreted in the urine, with a substantial portion of this being represented by the parent drug rather than metabolites. In humans, over 90% of the recovered drug-related material was unchanged varenicline, indicating that renal secretion of varenicline is a major route of drug clearance. This is consistent with clinical pharmacokinetic studies in which a small increase in varenicline exposure was observed with concomitantly administered cimetidine, a drug known to affect active secretion of drugs by the kidney, as well as moderately greater varenicline exposures in patients with renal impairment (Faessel, et al., unpublished). In other work using in vitro approaches, it has been demonstrated that while varenicline can readily penetrate biological membranes by passive diffusion, it is also a substrate for human organic cation transporter 2 (OCT-2), a renal transport protein involved in active secretion of cationic drugs (Feng, et al., manuscript in preparation). Thus, in combination, the presently reported excretion data, the aforementioned clinical pharmacokinetic data, and the in vitro studies all provide a consistent picture of the main mechanism of varenicline clearance as one of active and passive renal clearance.

Varenicline is subject to some metabolism, however metabolites are relatively few and are in low abundance. Metabolites observed in humans were also observed in animal species, and there was no readily observable difference between the smoker and

nonsmoker subjects. Circulating drug-related material was largely comprised of unchanged parent drug and the pharmacokinetics of total drug-related material and unchanged drug were very similar in each species. It should be noted that slightly differing values for the percentage of total circulating drug-related material comprised by varenicline were obtained when calculated from the radiometric HPLC profiles (Table 4) and the comparison of AUC values for unchanged drug and total radioactivity (Table 2). An explanation for this is not forthcoming, albeit the conclusion that varenicline comprises most of the total drug-related material is the same irrespective of the method of measurement. In human, the half-life of varenicline and total drug-related material was 17 hr in both cases.

Metabolites observed were primarily derived from reactions at the alicyclic nitrogen. Three conjugates, an N-carbamoylglucuronide (discussed below), an N-glucose adduct, and an N-formyl conjugate were observed. Such metabolites are likely due to the nucleophilic nature of this nitrogen. Formation of the N-glucose conjugate could be observed in a solution of varenicline and concentrated glucose without any catalyst. Whether this metabolite is generated by an enzyme catalyzed process in vivo or whether it forms spontaneously and reversibly with glucose is not known. We did not observe formation of this metabolite when incubated with liver microsomes supplemented with uridine diphosphoglucose (data not shown), despite the fact that UGT enzymes can sometimes catalyze glucosation reactions (Toide, et al., 2004; Senafi, et al., 1994). Also, attempts to observe the N-formyl conjugate in vitro were unsuccessful, so knowledge of the enzyme responsible for generation of this metabolite remains unknown.

However, both of these conjugates are very low in abundance and are not observed as excretory pathways.

In human, only two metabolites were observed in excreta: 2-hydroxyvarenicline and varenicline N-carbamoylglucuronide, each at about 3-4% of total. The N-carbamoylglucuronide was observed in vitro, under conditions required to observe this reaction (e.g. CO_2 atmosphere). UGT2B7 was shown to catalyze this reaction, which is similar to recently reported data for sertraline (Obach, et al., 2005). Whether the UGT enzyme catalyzes the association of the carbamic acid followed by conjugation with glucuronic acid, or whether the carbamic acid forms spontaneously and is then conjugated is not known. However, there is precedent for the spontaneous association/dissociation of carbamic acids in aqueous solutions (Caplow, 1968; Morrow, et al., 1974) and protophilic solvents (Masuda, et al., 2005), and since UGT2B7 is also known to catalyze the glucuronidation of carboxylic acids, it is not unreasonable to propose that it is the transiently formed varenicline carbamic acid that serves as the substrate. The other human excretory metabolite, 2-hydroxyvarenicline, was not observed in human in vitro systems active with cytochrome P450 or molybdenum cofactor oxidases, two families of enzymes that are known to catalyze such a reaction. With the metabolic clearance of varenicline being so low, it is not unexpected that this metabolic pathway would be challenging to study in vitro.

In summary, the overall disposition and metabolism of varenicline have been determined in humans and laboratory animal species. A few minor metabolites were observed and these were primarily derived from reactions occurring at the alicyclic nitrogen. However, the vast majority of drug-related material both in circulation and

excreta is represented by unchanged varenicline, which subsequently becomes excreted in the urine. This straightforward dispositional profile should simplify the use of this new agent in clinical practice.

References

Caplow M (1968) Kinetics of carbamate formation and breakdown. J. Am. Chem. Soc. 6795-6803.

Coe JW, Brooks PR, Vetelino MG, Wirtz MC, Arnold EP, Huang, J, Sands SB, et al., (2005) Varenicline: An α4β2 Nicotinic Receptor Partial Agonist for Smoking Cessation. J. Med. Chem. 48: 3474-3477.

Doll R, Peto R, Boreham J, Sutherland I (2005) Mortality from cancer in relation to smoking: 50 years observations on British doctors. Br. J. Cancer 92: 426-469.

Dow J, Piriou F, Wolf ED, Bertrand D, Haegele KD (1994) Novel carbamate metabolites of mofegiline, a primary amine monoamine oxidase B inhibitor, in dogs and humans. Drug Metab. Dispos. 22: 738-749.

Ezzati M and Lopez AD (2003) Estimates of global mortality attributable to smoking in 2000. Lancet 362: 847-852.

Hamilton RA, Garnett WR, Kline BJ (1981) determination of mean valproic acid serum level by assay of a single pooled sample Clin. Pharmacol. Ther. 29: 408-413.

Mackay J and Eriksen, M (2002) The Tobacco Atlas. World Health Organization http://www.who.int/tobacco/statistics/tobacco_atlas/en/ Accessed June 20, 2005.

Masuda K, Ito Y, Horiguchi M and Fujita H (2005) Studies on the solvent dependence of the carbamic acid formation from ω-(1-naphthyl)alkylamines and carbon dioxide. Tetrahedron 61: 213-229.

Morrow JS, Keim P, Gurd FRN (1974) CO_2 adducts of certain amino acids, peptides, and sperm whale myoglobin studied by carbon-13 and proton nuclear magnetic resonance. J. Biol. Chem. 249: 7484-7494.

Obach RS, Cox LM, Tremaine LM (2005) Sertraline is metabolized by multiple cytochrome P450 enzymes, monoamine oxidases, and glucuronyl transferases in human: an in vitro study. Drug Metab. Dispos 33: 262-270.

Schaefer WH. (1992) Formation of a carbamoyl glucuronide conjugate of carvedilol in vitro using dog and rat liver microsomes. Drug Metab. Dispos. 20: 130-133.

Senafi SB, Clarke DJ, Burchell B (1994) Investigation of the substrate specificity of a cloned expressed human bilirubin UDP-glucuronosyltransferase: UDP-sugar specificity

and involvement in steroid and xenobiotic glucuronidation. Biochem. J. 303: 233-240.

Taylor AL and Bettcher DW (2000) WHO Framework Convention on Tobacco Control: a global "good" for public health. Bull. World Health Organ. 78: 920-929.

Toide K, Terauchi Y, Fujii T, Yamazaki H, Kamataki T. (2004) Uridine diphosphate sugar-selective conjugation of an aldose reductase inhibitor (AS-3201) by UDP-glucuronosyltransferase 2B subfamily in human liver microsomes. Biochem. Pharmacol. 67: 1269-1278.

FIGURE 1. The structure of varenicline. The asterisks denote the position of carbon-14.

FIGURE 2. Recovery of varenicline related material in excreta after oral administration to mice, rats, monkey, and humans versus time. Symbols are: mouse (◊), rat ( ), monkey (Δ), and human (○).

FIGURE 3. Circulating concentrations of varenicline and total drug-related material in mice, rats, ,monkeys, and humans after oral administration of [^{14}C]varenicline. Varenicline ( ), total drug-related material (◊).

FIGURE 4. HPLC Radiochromatograms of Urine of Mice, Rats, Monkeys, and Humans After Oral Administration of [^{14}C]Varenicline.

FIGURE 5. HPLC Radiochromatograms of Plasma of Mice, Rats, Monkeys, and Humans After Oral Administration of [^{14}C]Varenicline.

FIGURE 6. Collision Induced Dissociation Mass Spectra for Varenicline and Metabolites. Panel A: Varenicline; Panel B: Metabolite M2 (amino acid); Panel C: 2-Hydroxyvarenicline; Panel D: Metabolite 3C; Panel E: Varenicline N-Carbamoylglucuronide; Panel F: N-Formyl Varenicline; Panel G: N-Glucosyl Varenicline.

FIGURE 7. HPLC-NMR Spectra of Varenicline, Metabolite 3b and an Authentic Standard of 2-Hydroxyvarenicline

FIGURE 8. Enzyme kinetics of N-carbamoylglucuronidation of varenicline in human liver microsomes (❁) and recombinanat human UGT2B7 (⑦).

FIGURE 9. Metabolism of Varenicline in Animals and Humans

TABLE 1. Material Balance and Routes of Excretion of [^{14}C]Varenicline Drug Related Material in Mice, Rats, Monkeys, and Humans.

Species	% of Dose in Urine	% of Dose in Feces	% of Dose Recovered[b]
Mouse	83.1 ± 8.2	10.8 ± 5.1	94.4 ± 6.7
Rat	68.1 ± 10.2	22.3 ± 6.3	90.4 ± 6.4
Monkey	74.1 ± 2.3	6.5 ± 0.5	87.5 ± 5.0
Human	87.1 ± 5.5	0.9 ± 0.5	88.0 ± 5.7

[a]Values represent mean +/- SD.

[b]Percentage of dose recovered also includes cage washes.

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TABLE 2. Pharmacokinetics of Varenicline and Total Radioactivity in Mice, Rats, Monkeys, and Humans After Oral Administration of [^{14}C]Varenicline.[a]

	Mouse	Rat	Monkey	Human
Dose (mg/kg)	3.0	3.0	0.08	1.0[b]
Varenicline:				
AUC(0-t_{last}) (ng•hr/ml)[d]	963	2390 ± 610	210 ± 26	90.3 ± 6.8
C_{max} (ng/ml)	293	234 ± 55	19.3 ± 3.4	4.01 ± 0.71
T_{max} (hr)	1.0	1.8 ± 1.3	3.0 ± 1.0	4.3 ± 2.3
$t_{1/2}$ (hr)	1.4	4.0 ± 0.9	24 ± 6	17 ± 3
CL/F (ml/min/kg)	52	21 ± 5	6.3 ± 0.8	2.5 ± 0.2
Total Radioactivity:				
AUC(0-t_{last}) (ngeq•hr/ml)[cd]	1380	4340 ± 980	331 ± 34	114 ± 11
C_{max} (ngeq/ml)[c]	386	413 ± 82	29.0 ± 6.4	4.57 ± 0.47
T_{max} (hr)	1.0	2.4 ± 1.4	3.0 ± 1.0	2.8 ± 1.6
$t_{1/2}$ (hr)	1.8	5.1 ± 0.6	30 ± 3	17 ± 2
Varenicline as % of total drug-related material	70	55	63	79

[a]Values represent Mean +/- SD

[b]Human dose was 1.0 mg, unadjusted for body weight.

[c]ngeq: nanogram equivalents of varenicline-related material.

[d]t_{last} values were 12 h (mouse), 24 h (rat, monkey), and 72 h (human).

TABLE 3. Quantitation of Varenicline and Major Metabolites in Excreta of Mice, Rats, Monkeys, and Humans After Oral Administration of [^{14}C]Varenicline.

Metabolite ($M+H^+$ ion)	Mouse	Rat	Monkey	Human
	% of dose			
M1 (amino acid metabolite; m/z 244)	0.53	0.86	1.1	ND
M2 (amino acid metabolite; m/z 244)	0.97	0.72	4.3	ND
M3 unknown (m/z 338)	ND	0.95	ND	ND
M3a unknown	0.12	ND	1.1	ND
M3b (2-Hydroxyvarenicline; m/z 228)	ND	ND	ND	2.9
Varenicline (m/z 212)	90	84	75	81
M4 (Varenicline N-Carbamoylglucuronide; m/z 432)	ND	1.9	3.6	3.6

[a]ND not detected

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TABLE 4. Quantitation of Varenicline and Major Metabolites in Circulation of Mice, Rats, Monkeys, and Humans After Oral Administration of [^{14}C]Varenicline

Metabolite ($M+H^+$ ion)	Mouse	Rat	Monkey	Human
	% Circulating Radioactivity[a]			
M1 (amino acid metabolite; m/z 244)	1.4	ND	ND	ND
M2 (amino acid metabolite; m/z 244)	3.8	0.7	1.7	ND
M3a unknown	1.1	ND	0.53	ND
M3c (putative lactam; m/z 226)	ND	ND	0.93	1.1
Varenicline (m/z 212)	85	81	80	91
M3d (putative lactam; m/z 226)	6.8	ND	2.1	ND
M4 (Varenicline N-Carbamoylglucuronide; m/z 432)	ND	11	8.7	3.8
M5 (N-Formylvarenicline; m/z 240)	2.5	5.3	2.6	0.9
M6 unknown	ND	1.7	ND	ND
M7 (N-Glucosylvarenicline; m/z 374)	ND	1.2	2.9	3.5

[a]ND not detected

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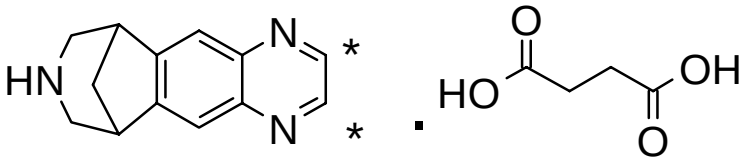

[^{14}C]varenicline succinate

Figure 1

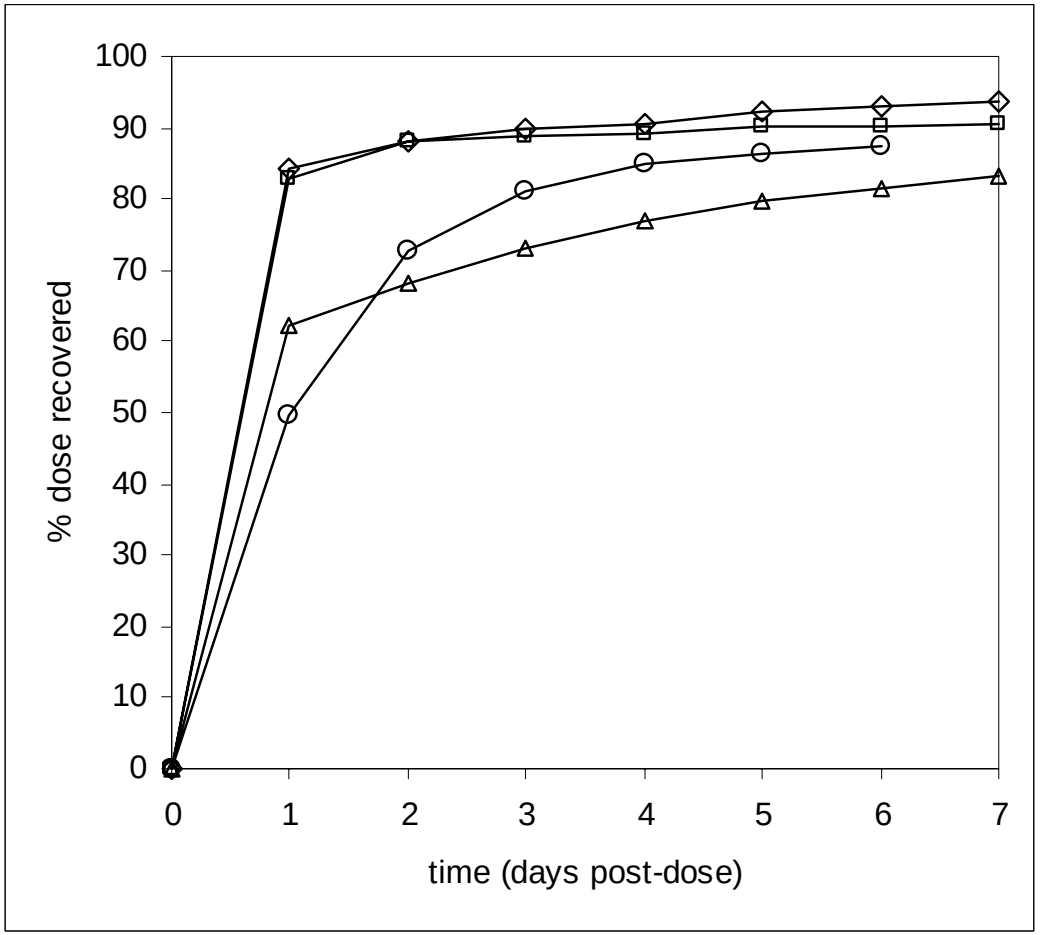

Figure 2

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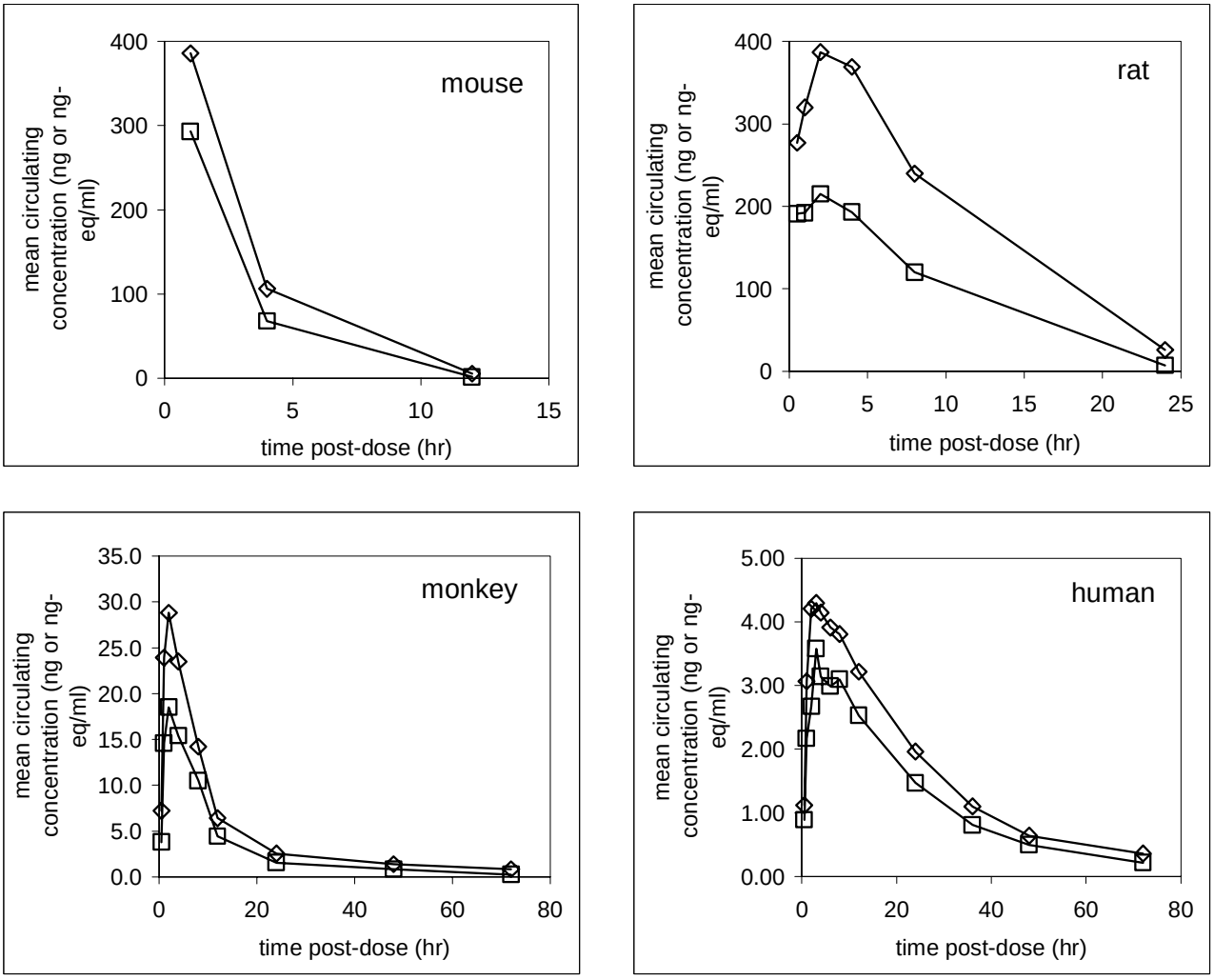

Figure 3

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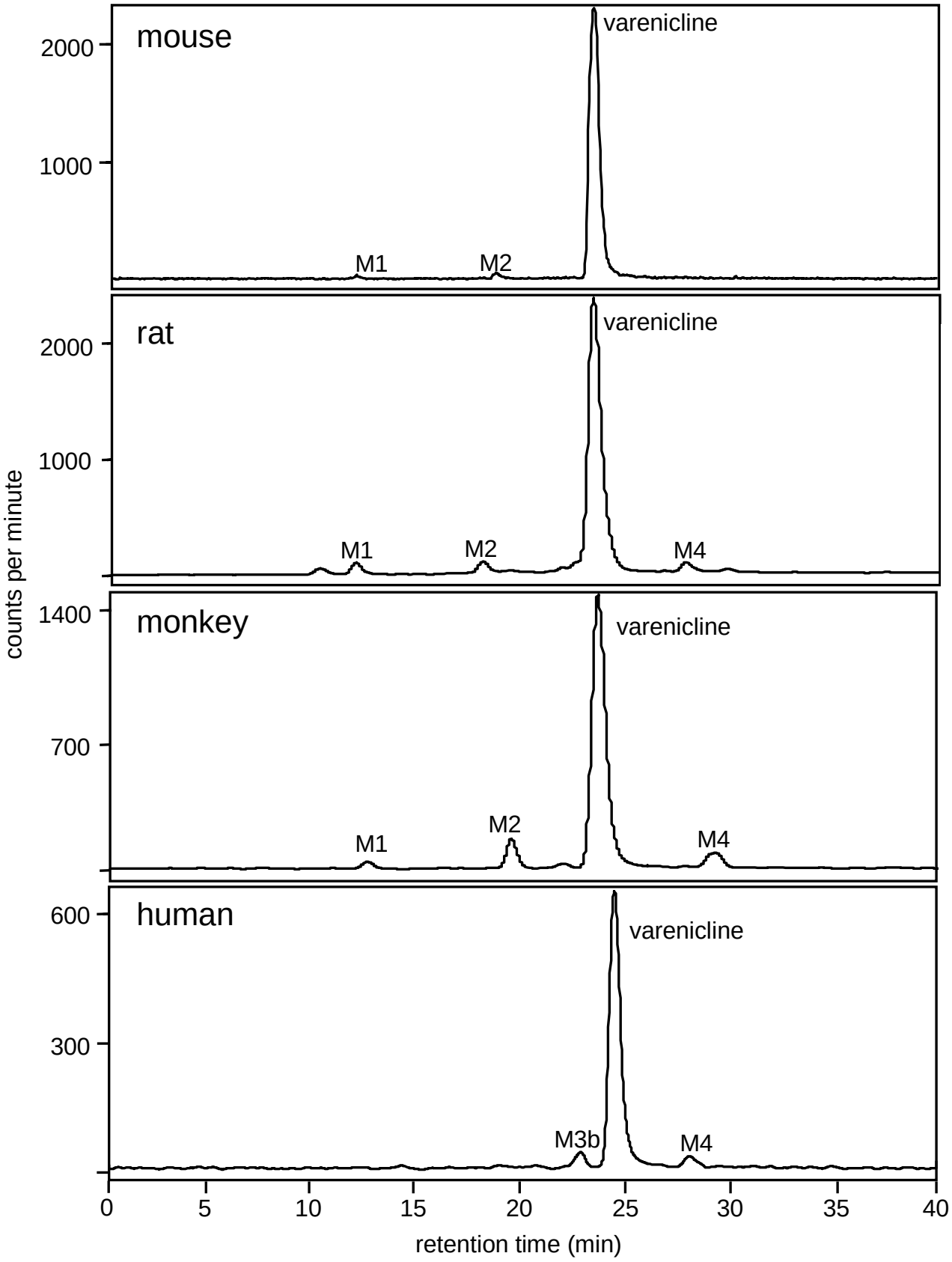

Figure 4

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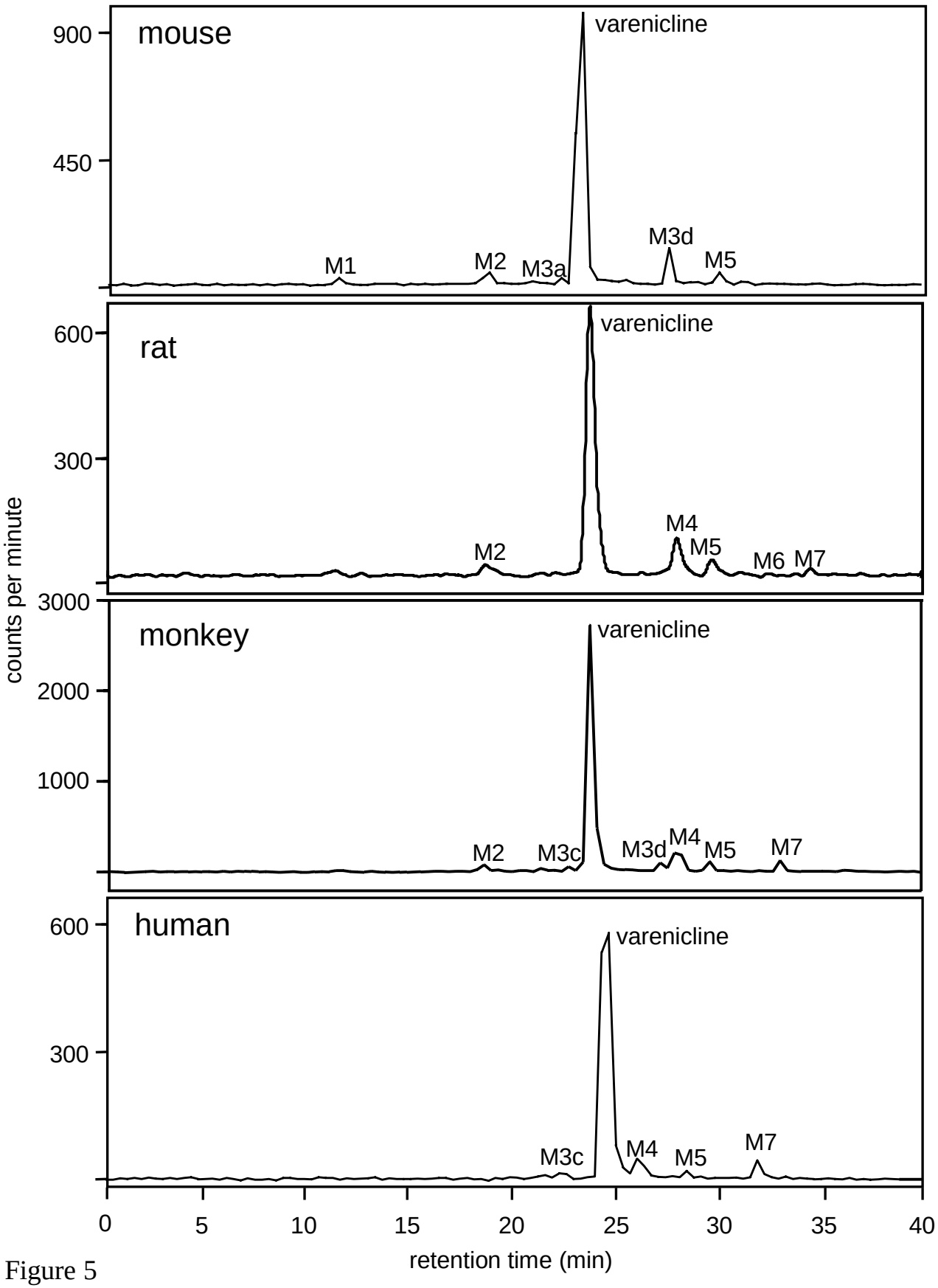

Figure 5

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Fig 6

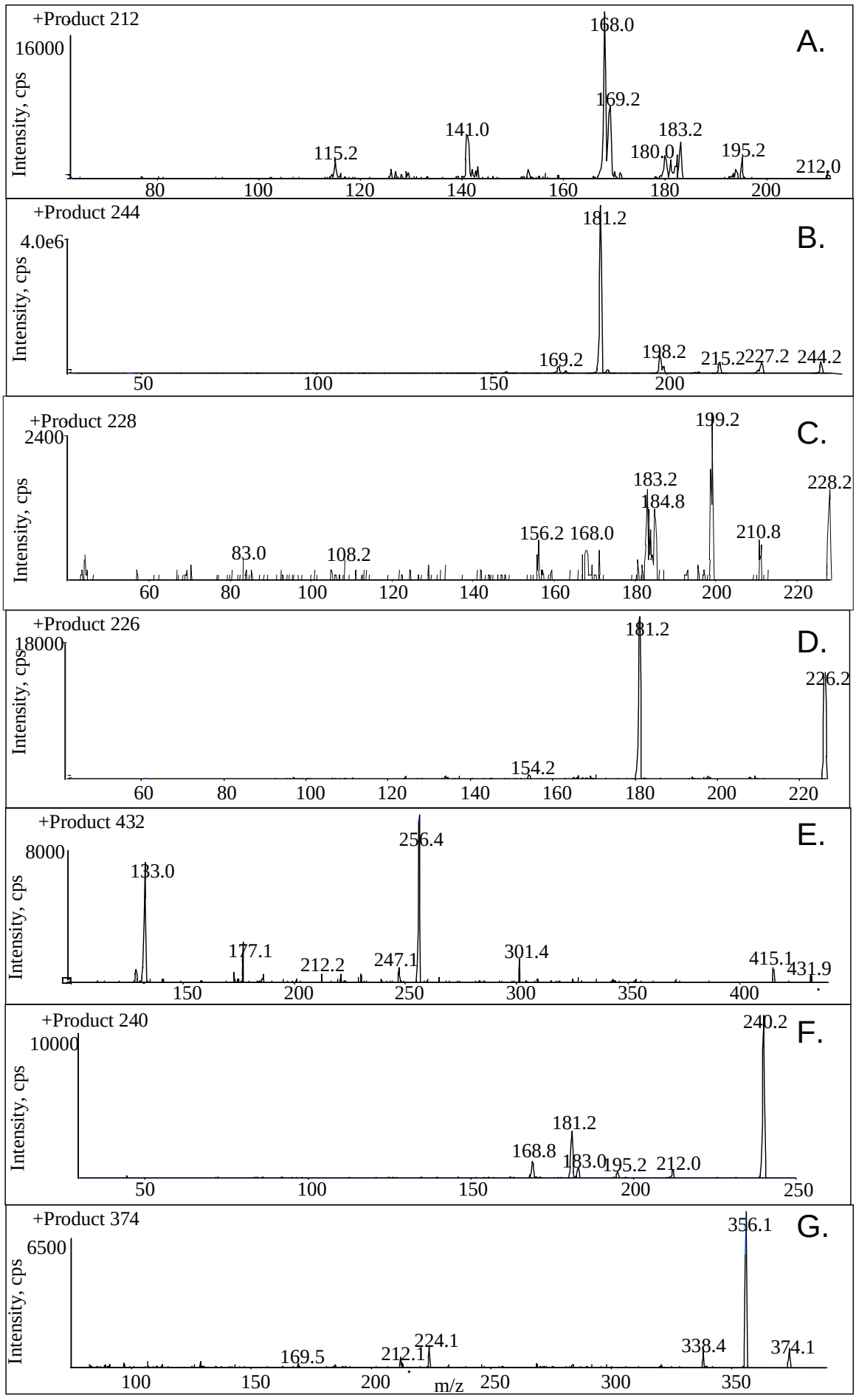

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Figure 7

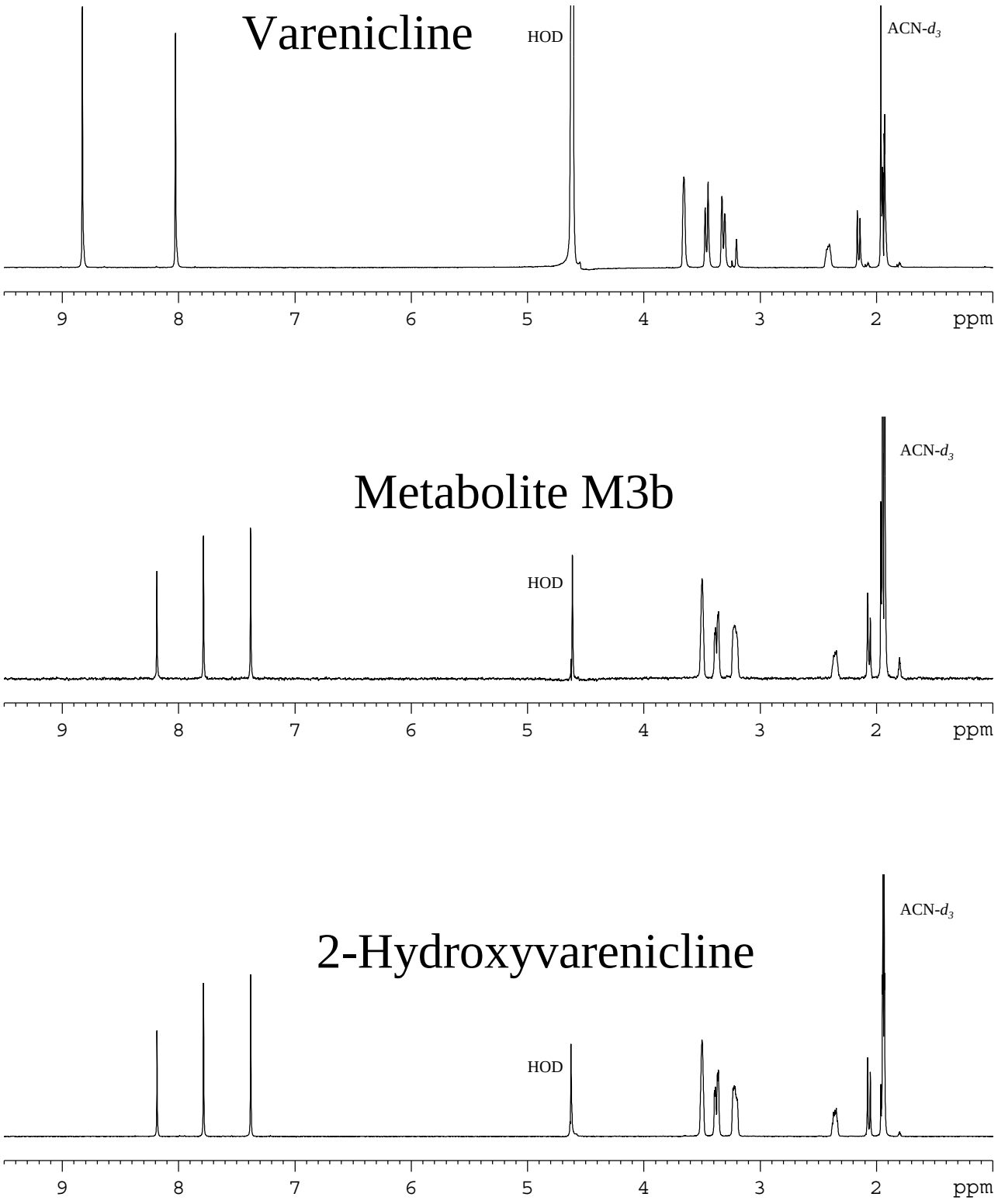

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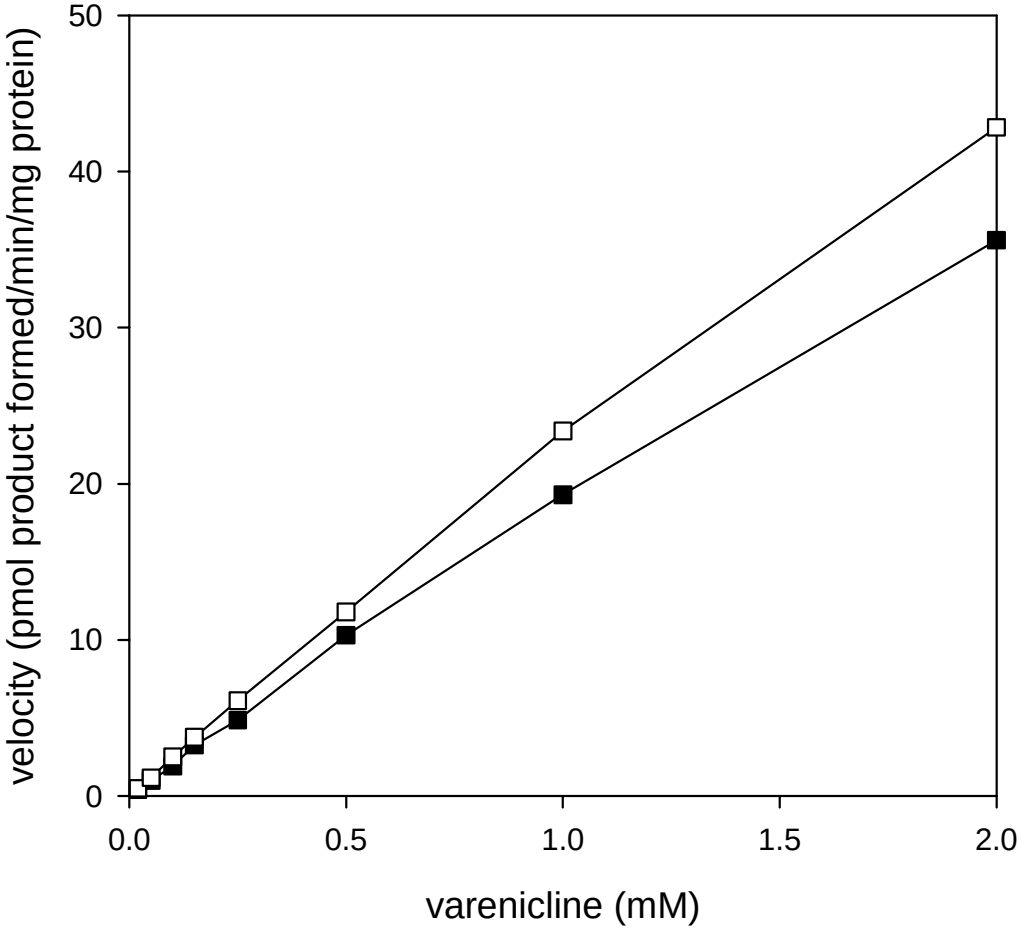
Figure 8



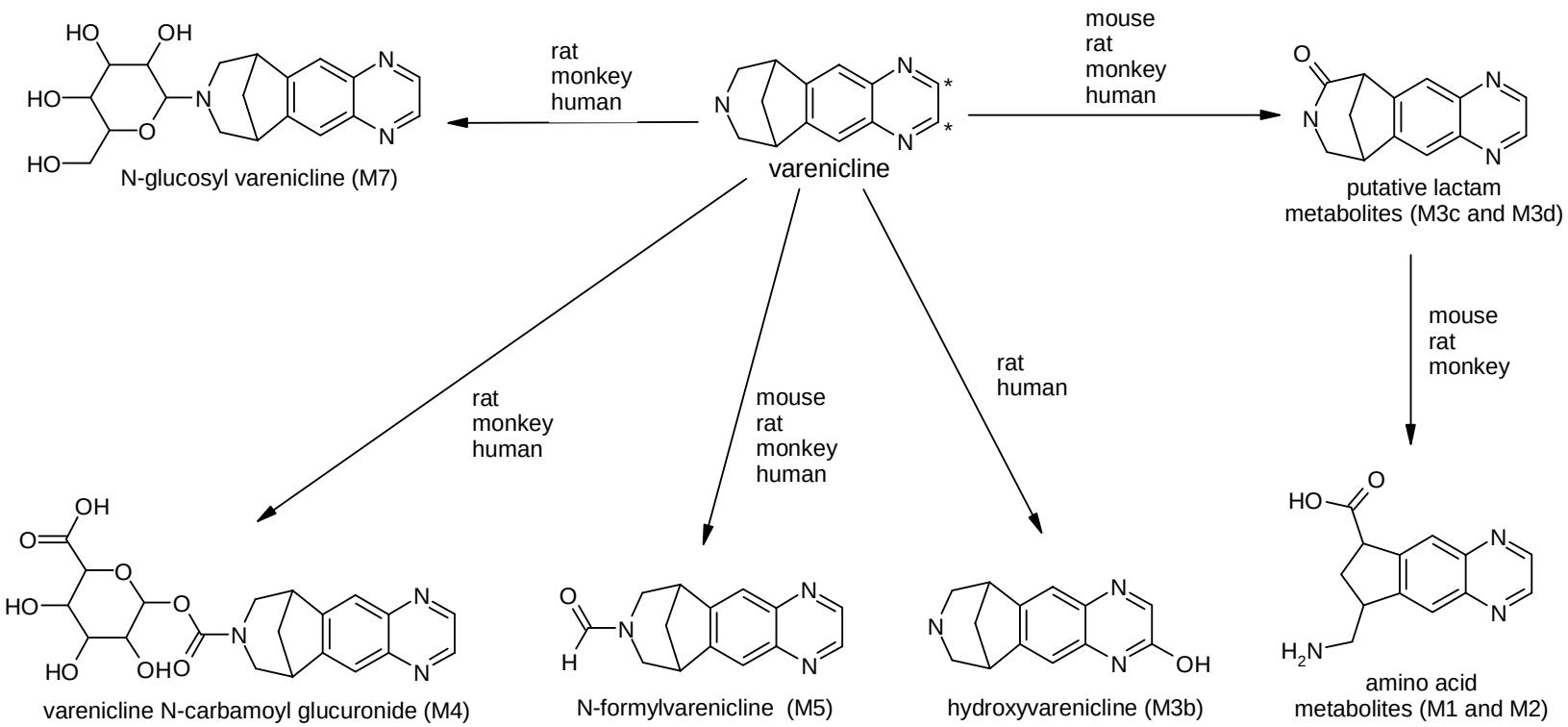


Figure 9