BIOTRANSFORMATION OF NEVIRAPINE, A NON-NUCLEOSIDE HIV-1 REVERSE TRANSCRIPTASE INHIBITOR, IN MICE, RATS, RABBITS, DOGS, MONKEYS, AND CHIMPANZEES¹

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ABSTRACT:
The study objectives were to characterize the metabolism of nevirapine (NVP) in mouse, rat, rabbit, dog, monkey, and chimpanzee after oral administration of carbon-14-labeled or -unlabeled NVP. Liquid scintillation counting quantitated radioactivity and bile, plasma, urine, and feces were profiled by HPLC/UV diode array and radioactivity detection. Metabolite structures were confirmed by UV spectral and chromatographic retention time comparisons with synthetic metabolite standards, by β-glucuronidase incubations, and in one case, by direct probe electron impact ionization/mass spectroscopy, chemical ionization/mass spectroscopy, and NMR. NVP was completely absorbed in both sexes of all species except male and female dogs. Parent compound accounted for <6% of total urinary radioactivity and <5.1% of total fecal radioactivity, except in dogs where 41 to 46% of the radioactivity was excreted as parent compound. The drug was extensively metabolized in both sexes of all animal species studied. Oxidation to hydroxylated metabolites occurred before glucuronide conjugation and excretion in urine and feces. Hydroxylated metabolites were 2-, 3-, 8-, and 12-hydroxynevirapine (2-, 3-, 8-, and 12-OHNVP). 4-carboxynevirapine, formed by secondary oxidation of 12-OHNVP, was a major urinary metabolite in all species except the female rat. Glucuronides of the hydroxylated metabolites were major or minor metabolites, depending on the species. Rat plasma profiles differed from urinary profiles with NVP and 12-OHNVP accounting for the majority of the total radioactivity. Dog plasma profiles, however, were similar to the urinary profiles with 12-OHNVP, its glucuronide conjugate, 4-carboxynevirapine, and 3-OHNVP glucuronide being the major metabolites. Overall, the same metabolites are formed in animals as are formed in humans.

The elimination of nevirapine (NVP, 5,11-dihydro-11-cyclopropyl-4-methyl-6H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6-one, Fig. 1)² via hepatic biotransformation and renal excretion was studied in mice, rats, rabbits, dogs, cynomolgus monkeys, and chimpanzees. The biotransformation pathways involved in its metabolism were determined and compared among the various species. NVP was the first of a new class of antiretroviral agents, the non-nucleoside HIV-1 reverse transcriptase inhibitors, to be approved by the Food and Drug Administration for marketing. It has subsequently been approved in Europe and other areas of the world. It is indicated for use in combination with other antiretroviral drugs, including nucleoside analogs and protease inhibitors, for the treatment of HIV-1 infected adults and children who have experienced clinical and/or immunological deterioration. NVP is a potent and selective inhibitor (IC₅₀ = 10–100 nM) of the replication of a wide variety of HIV-1 strains in several cellular assays (Merluzzi et al., 1990). It is a noncompetitive inhibitor that binds at a site on the reverse transcriptase enzyme separate from the substrate binding site (Wu et al., 1991; Kohlstaedt et al., 1992). When studied in vitro in combination with nucleoside reverse transcriptase inhibitors, the combined antiviral activity is either additive or synergistic (Koup et al., 1993). NVP, administered in combination with two other antiretroviral drugs, offers a significant benefit based on surrogate marker responses in clinical trials of greater than 2 years duration (Montaner et al., 1998a,b). The drug is active without metabolic processing, greater than 90% absorbed, and widely distributed throughout the body. It has a long half-life, making feasible twice daily (b.i.d.) administration of a 200-mg tablet and, therefore, easy compliance (Murphy and Montaner, 1996). During development, the metabolism of NVP was compared across species to understand the fate of the drug and to verify that the toxicology species (rat and dog) were exposed to the same metabolites as individuals undergoing treatment.
Radiolabeled NVP contained carbon-14 in the carbonyl position. It has a pKa of 2.8, melting point of 228–230°C and lipophilicity (log D) of 1.92. Radiolabeled NVP contained carbon-14 in the carbonyl position (+).

Materials and Methods

Chemicals. NVP has a molecular weight of 266.302 and an empirical formula of C15H14N4O. Radiolabeled NVP, containing carbon-14 in the C-6 carbonyl position, was synthesized by a four-step procedure from barium [14C]-carbonate at Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT). The radiolabeled compound was 93.9% radiopure by HPLC and had a specific activity of 90.9 μCi/mg (25.1 mCi/mmol). Authentic samples of 2-, 3-, 8-, and 12-hydroxynevirapine (2-, 3-, 8-, and 12-OHNVP) and 4-carboxynevirapine (4-CANVP) were also synthesized at Boehringer Ingelheim Pharmaceuticals, Inc.

Animals. Male and female C57BL/6J mice (21–24 g, 10 each sex) and three female rhesus monkeys of the Himalayan strain, Cribb:HM (2.5–2.6 kg), bred at Dr. Karl Thomae Laboratories, GmbH (Biberach an der Riss, Germany), were obtained from the breeding facility. Male and female (two per sex) Sprague-Dawley Taconic males and females were obtained from Taconic Farms, Germantown, NY. Male and female beagle dogs (10.6–11.8 kg, one male and two females) were obtained from White Eagle Laboratories (Dayestown, PA) or Marshall Farms (North Rose, NY). Male (n = 2) and female (n = 1) cynomolgus monkeys were purchased from Charles River Primate Imports (Wilmington, MA; Yale University, New Haven, CT; or Buckshire Corp., Perkasie, PA). Three female chimpanzees were purchased from and maintained by The Laboratory for Experimental Medicine and Surgery in Primates (Tuxedo, NY).

Dosing of Animals. Animals were fasted for 15 h before dose administration. For oral administration, males were fasted ad libitum. Mice were fasted for 15 h before dose administration. Oral dosing to rats, dogs, and monkeys was carried out at Dr. Karl Thomae Laboratories, GmbH. To prepare the dosing suspensions for oral administration to rats, dogs, and monkeys, NVP and [14C]NVP were dissolved together in absolute ethanol and methylene chloride (1:1, v/v) with mild heating. The mixture was concentrated by rotary evaporation, vacuum dried at 40°C, and added to 0.5% natsol (250 HX; hydroxyethylcellulose prepared in distilled water). It was suspended by stirring overnight at 4°C and sonication in an ultrasonic bath. The concentration of drug in suspension was 2 mg/ml (20 mg/kg, 2.5 μCi) with a specific activity of 5.55 μCi/mg for oral dosing to mice and 6 mg/ml (36 mg/kg, 92 μCi) with a specific activity of 1.01 μCi/mg for oral dosing to rabbits. Dosing and sample collection in mice and rabbits was carried out at Dr. Karl Thomae Laboratories, GmbH. To prepare the dosing suspensions for oral administration to rats, dogs, and monkeys, NVP and [14C]NVP were dissolved together in absolute ethanol and the ethanol was evaporated overnight in a fume hood. The dried material was suspended in 0.5% methylecellulose (Methocel E4 M Premium, Dow Chemical Co.) with sonication. The concentration of drug in suspension was 20 mg/ml (20 mg/kg, 228 μCi) with a specific activity of 1.03 μCi/mg for dosing to dogs. A concentration of 5 mg/ml (20 mg/kg, 3.3 μCi) with a specific activity of 0.87 μCi/mg was prepared for dosing to monkeys. The concentration of drug in suspension was 2 mg/ml (20 mg/kg, 26 μCi) for oral dosing to rats and 6.7 mg/ml (20.3 mg/kg, 10 μCi males, 8.9 μCi females) for intraduodenal administration to rats before bile collection. The i.v. dose was administered to rats (1.1 mg/kg, 20 μCi) as a solution in 20% ethanol/80% saline. Dosing and sample collection in rats, dogs, and monkeys was carried out at Boehringer Ingelheim Pharmaceuticals, Inc. Female chimpanzees were sedated with 10 to 15 mg/kg ketamine and, in a supine position, administered 25.7 mg/kg unlabeled NVP in 0.5% methylecellulose suspension at The Laboratory for Experimental Medicine and Surgery in Primates.

Urine and Fecal Sample Collection. After dose administration, mice, rats, rabbits, dogs, and monkeys were placed in metabolism cages and their urine and feces were collected on ice. Samples from mice and rabbits were collected over the following timed intervals: 0 to 8, 8 to 24, 24 to 48, 48 to 72, and 72 to 96 h. Samples from rats, dogs, and monkeys were collected over the timed intervals 0 to 24, 24 to 48, 48 to 72, and 72 to 96 h. Urine samples were collected from chimpanzees at 12- and 24-h intervals after dose administration. Urine samples were counted in duplicate for radioactivity, and fecal samples were counted in triplicate.

Urine and Fecal Sample Preparation. Fifty percent of the rat urine from the 0- to 24-h collection interval was pooled for analysis. Samples from males and females were pooled separately. Ten percent of each urine sample from mice, rabbits, dogs, and monkeys were pooled with the sexes pooled separately. Urine samples from chimpanzees were analyzed directly without pooling.

Fifty percent of each 0- to 24-h fecal sample from male rats was pooled for analysis and fifty percent of each 0- to 24- and 24- to 48-h fecal sample from female rats was pooled for the analysis. Five percent of each 0- to 24-h fecal sample from male and female dogs was pooled separately by sex with an additional 0- to 48-h fecal sample pool prepared from females. Five percent of each 0- to 24-h fecal sample volume from rabbits and monkeys was taken for analysis, and 10% of each 0- to 8- and 8- to 24-h fecal sample from mice was pooled for analysis. All urine and fecal sample pools were centrifuged for 15 min at 2000 rpm and the supernatants were decanted for preparation and analysis. Aliquots (10 μl) of each sample pool were counted in a Beckman LS 5000 TA (Beckman Instruments Inc., Fullerton, CA) for the determination of radioactivity. After centrifugation 90% or more of the radioactivity was determined to be in the supernatant of all the species.

Collection of Plasma and Bile Samples. An additional four rats and four dogs (two males and two females of each species) were administered 20 mg/kg [14C]NVP by oral gavage. Plasma samples were collected from male and female rats by retro-orbital puncture 2 and 4 h after administration, respectively. Plasma samples were collected from male and three female rats by cannulation of the ductus choledochus after administration of radiolabeled drug by jugular cannulation over the timed intervals at 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 6, and 6 to 24 h. A 100-μl aliquot of the sample from each time interval was pooled for each sex. Plasma and bile sample pools were vortexed thoroughly and 10-μl aliquots of each sample pool were counted in a Beckman LS 5000 TA (Beckman Instruments Inc., Fullerton, CA) for the determination of radioactivity.

Sample Preparation and Analysis. Samples were prepared by solid phase extraction (SPE) using a Visiprep 24 Port-DL Solid Phase Extraction Manifold (Supelco, Bellefonte, PA) equipped with a Supelco SPE Vacuum Pump Trap Kit attached to a Welch GEM 8890 vacuum pump (Welch Vacuum Pump Technology Inc., East Hanover, NJ) to provide continuous vacuum. Urine, feces, or plasma were individually loaded onto activated Sep-Pak Vac 6 cc (1 g) C18 Cartridges (Waters Corp., Milford, MA) and slowly eluted through the cartridge in a dropwise fashion. The cartridges were washed with 5 ml of Omnisolv HPLC water (EM Science, Gibbstown, NJ); the entire aqueous portion was collected and the cartridge was allowed to dry under vacuum. Finally, the cartridges were eluted with 3 ml of methanol and dried under nitrogen at 40°C in a Zymark Turbo Vap LV Evaporator (Zymark Corp., Hopkinton, MA). The dried urine, feces, and plasma extracts were taken up in 200 μl of 0.05 M KH2PO4 with 0.1% triethylamine (v/v). Exceptions were for male rat and male dog urine after p.o. administration, which were taken up in 37.5 μl of methanol. Samples were then sonicated and vigorously vortexed. Sample radioactivity was counted after every step of each SPE procedure to determine the extraction efficiency.

Metabolite Profile Analysis. Radiolabeled (1 mg) and nonlabeled NVP (4 mg) were mixed and dissolved in methanol to yield a 1 μg/μl standard stock solution. From this solution, mobile phase standards were made with concentrations ranging from 0.05 to 50 μg/ml. The specific activity of these standards was 34.2 μCi/mg (9.1 μCi/mmol). The standards were used for the generation of parent compound radiochromatograms and served as HPLC performance standards. The urine and fecal sample pools volumes associated with 200,000
After administration of a single oral (20 mg/
kg) or i.v. (1 mg/kg) dose of radiolabeled NVP, most species excreted approximately twice as much radiolabel in urine as in feces. Exceptions were the male rat that excreted equal radiolabel in urine and feces, and dogs where absorption was poor. In this species, the majority of the label was excreted in the feces, as shown in Table 1.

Comparison of Mouse, Rat, Rabbit, Beagle Dog, and Cynomolgus Monkey Urine Metabolite Patterns. The metabolite patterns of the 0- to 24-h urine extracts after p.o. administration of [14C]NVP in males of all animal species except rabbits are shown in Fig. 2. The profiles demonstrated extensive metabolism of [14C]NVP. A single male cynomolgus monkey exhibited the greatest quantity of parent compound (56%; a second male excreted no parent) whereas it was barely detectable in the other species. 4-CANVP appeared as a major metabolite in all species. Additionally, 2-OHNV glucuronide and

Fig. 2. Radiochromatographic overlays of the 0- to 24-h urine extracts from male mice (A), rats (B), beagle dogs (C), and cynomolgus monkeys (D and E) after oral administration of [14C]NVP at 20 mg/kg.
3-OHNVP glucuronide were major metabolites in all species except the rat. Glucuronidation was most prevalent in the dog and monkey (dog > monkey > mouse > rat). Finally, several identified and unknown minor metabolites were distributed throughout the patterns in each species, as shown in Table 2 and Fig. 2.

The corresponding urine metabolite patterns in female animals after p.o. administration of \(^{14}C\)NVP are displayed in Fig. 3. Like the male, the female animals of all species demonstrated extensive metabolism of \(^{14}C\)NVP. 4-CANVP appeared as a major metabolite in the mouse, dog, and monkey, whereas the M9/M10/M11 complex of...
unidentified metabolites accounted for the major portion of the radio-label in the female rat and for a minor portion in the rabbit. The glucuronide metabolites, 2-OHNVP glucuronide and 3-OHNVP glucuronide, were major metabolites in the mouse, rabbit, dog, and monkey. In the rat, these glucuronides were minor metabolites. 12-OHNVP glucuronide was found as a major or minor urinary metabolite in all species except the mouse. Again, several other identified and unidentified minor metabolites were present in the urine from females of all species.

**Comparison of Mouse, Rat, Rabbit, Beagle Dog, and Cynomolgus Monkey Fecal Metabolite Patterns.** Figures 4 and 5 display the corresponding radiochromatograms of the male and female 0- to 24- and 0- to 48-h (female dog only) fecal metabolite patterns, respectively. The glucuronide conjugates were found in low quantities or...
were not detectable in both sexes and all species. 4-CANVP was a major metabolite in all the male animals and the female mouse, dog, and monkey. In the female rat and rabbit, the unidentified M9/M10/M11 complex accounted for a major portion of the radiolabel. 3-OHNVP was a major fecal metabolite in all animals except for the male rat. As in the urine, several identified and unidentified minor metabolites were present in the fecal metabolite patterns of each sex and species. [14C]NVP was present in low quantities in the feces of all animals (<5.1%) except in the male and female dog. The percentages of [14C]NVP and known metabolites are displayed in Table 2.

Comparison of Male and Female Rat Metabolite Patterns in Bile. Figures 6 and 7 display representative 0- to 24-h bile radiochromatograms after i.v. administration to male and female rats. Similar to the urine of both sexes, the bile profiles also demonstrated extensive oxidative metabolism of [14C]NVP. The major metabolites in the male bile were 4-CANVP and 12-OHNVP glucuronide. The female bile major metabolites were 12-OHNVP glucuronide, 2-OHNVP glucuronide, and 3-OHNVP glucuronide. 2-OHNVP, 12-OHNVP, 8-OHNVP glucuronide, 2-OHNVP glucuronide, and 3-OHNVP glucuronide were minor metabolites in the male, whereas 12-OHNVP, 8-OHNVP glucuronide, and the M9/M10/M11 complex were minor metabolites in the female. Very small quantities of [14C]NVP were found. The summary of the percentages of the total radioactivity excreted in bile as each metabolite is displayed in Table 2.

Comparison of Male and Female Rat Metabolite Patterns in Plasma. The radiochromatograms of plasma extracts from male and female rats 2 and 4 h after p.o. administration of [14C]NVP are shown in Fig. 8. At 2 h, [14C]NVP represented 34.8% of the total radioactivity, and 12-OHNVP was the primary major metabolite in the male. The additional metabolites found were 12-OHNVP glucuronide, 2-OHNVP glucuronide, 3-OHNVP glucuronide, and the 2-OHNVP/M9 coeluting peak. Unlike the male, [14C]NVP represented the majority of the radioactivity (82%) in female rat plasma. 12-OHNVP was again the
major metabolite. Minor metabolites were 12-OHNVP glucuronide, 2-OHNVP glucuronide, 3-OHNVP glucuronide, and the 2-OHNVP/M9/M11 coeluting peak. The tabulation of the percentages of the radioactivity excreted as NVP and known plasma metabolites in both sexes is displayed in Table 2.

**Gender Comparison of Urine, Fecal, and Bile Metabolite Patterns in Rats.** Figures 6 and 7 display the representative radiochromatographic overlays of the 0- to 24-h urine, fecal (female = 0–48 h), and bile extracts, respectively, from male and female rats after i.v. administration of [14C]NVP at 20 and 36 mg/kg (rabbits only).
Fig. 6. Radiochromatographic overlay of the representative 0- to 24-h urine (A), feces (B), and bile (C) extracts from male rats after i.v. administration of [14C]NVP at 1 mg/kg.

The female rat formed three unidentified metabolites eluting at approximately 27.5 min that displayed different UV spectra than 4-CANVP, the major metabolite in male rat.

Fig. 7. Radiochromatographic overlay of the representative 0- to 24-h urine (A), 0- to 48-h feces (B), and 0- to 24-h bile (C) extracts from female rats after i.v. administration of [14C]NVP at 1 mg/kg.

The female rat formed three unidentified metabolites eluting at approximately 27.5 min that displayed different UV spectra than 4-CANVP, the major metabolite in male rat.
uronide. The glucuronide metabolites were not found in the feces. 12-OHNVP was a minor metabolite in all the matrices. [14C]NVP was barely detectable in all three matrices.

In the female the glucuronide conjugates (12-OHNVP glucuronide, 2-OHNVP glucuronide, and 3-OHNVP glucuronide) also did not appear in the feces but were major metabolites in bile and minor metabolites in urine. The M9/M10/M11 complex of metabolites accounted for a major portion of radiolabel in the urine, whereas 12-OHNVP and 4-CANVP were major metabolites in feces. 12-OHNVP was found to be a major metabolite only in the urine. As in the male, [14C]NVP appeared as a small percentage of the total pattern.

Comparison of Male and Female Dog Metabolite Patterns in Plasma. Due to the low level of radioactivity in dog plasma, UV chromatograms (λ = 240 nm) were used for quantitation of the metabolite patterns in dog plasma. The plasma chromatograms contained little endogenous background matrix interference with metabolites or parent compound. The metabolites and [14C]NVP were assumed to have approximately the same extinction coefficients for the purposes of quantitation. At Tmax, NVP represented 16.2% of the plasma analyte signal and 12-OHNVP, 4-CANVP, 12-OHNVP glucuronide, and 3-OHNVP glucuronide were the major metabolites. The minor metabolites were 3-OHNVP and 12-OHNVP. The major difference between the plasma and urine metabolite patterns was the absence of 2-OHNVP glucuronide in the plasma and the absence of 3-OHNVP and 12-OHNVP in the urine.

The female plasma metabolite patterns were qualitatively similar to those observed in the male. NVP represented 10.1% of the total plasma analyte signal at Tmax and, like the male, the major metabolites were 12-OHNVP, 4-CANVP, 12-OHNVP glucuronide, and 3-OHNVP glucuronide. Minor metabolites were 3-OHNVP and 8-OHNVP glucuronide. The difference between the plasma and urine metabolite patterns was identical with the difference observed in the male patterns. Table 2 summarizes the percentages of [14C]NVP and known metabolites in the total plasma analyte signal.

Isolated 12-OHNVP Glucuronide, 2-OHNVP Glucuronide, and 3-OHNVP Glucuronide β-glucuronidase Incubation. Because 2-OHNVP glucuronide and 3-OHNVP glucuronide were not completely separated chromatographically, it was impossible to quantitate them individually. To accomplish this, the combined metabolite peak was isolated from dog urine, hydrolyzed, and the aglycones were quantitated for additional information on composition. In male dogs, the 2-OHNVP glucuronide/3-OHNVP glucuronide peak was composed of 19% 2-OHNVP glucuronide and 81% 3-OHNVP glucuronide.

### Table 3

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<th>Chimpanzee ID</th>
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<th>M4</th>
<th>M6</th>
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nide. In female dogs, the aglycon metabolite composition was almost identical with the male (2-OHNVP glucuronide = 20% and 3-OHNVP glucuronide = 80%). Enzyme hydrolysis of 12-OHNVP glucuronide liberated 12-OHNVP in samples from both sexes.

**Female Chimpanzee Urinary Metabolite Patterns.** The UV chromatograms (λ = 240 nm) of the 0- to 12- and 12- to 24-h urine metabolite patterns after oral administration to three female chimpanzees indicate that NVP was extensively metabolized at each time point in chimpanzees, as in other species. In the 0- to 12-h chromatograms, NVP represented a small percentage of the metabolite profiles except in chimpanzee no. 440. In the 12- to 24-h chromatograms, NVP also represented small percentages of the metabolite profiles except for chimpanzee no. 418.

The major metabolites in the 0- to 12-h metabolite profiles were the
12-OHNVP, 4-CANVP, 12-OHNVP glucuronide, 2-OHNVP glucuronide, and 3-OHNVP glucuronide. All other metabolites were minor metabolites representing less than 10% of the metabolite pattern (e.g., 3-OHNVP, 12-OHNVP, and 12-OHNVP glucuronide). In the 12- to 24-h urine metabolite patterns, 2-OHNVP glucuronide and 3-OHNVP glucuronide were major metabolites and greatly increased in percentage in chimpanzee nos. 440 and 454. 12-OHNVP glucuronide was a major metabolite in chimpanzee nos. 440 and 454. 4-CANVP was present as a major metabolite only in no. 440. As in the 0- to 12-h urine profiles, several minor metabolites were present (i.e., 3-OHNVP, 12-OHNVP, 8-OHNVP glucuronide, and 4-CANVP). The percentages of NVP and known metabolites in the urine metabolite patterns are displayed in Table 3. The calculation of these percentages was based on the assumption that parent compound and metabolites have identical extinction coefficients. Synthetic standards of all the metabolites were not available for the exact determination of metabolite percentages using appropriate calibration curves. Preliminary calculations, however, demonstrated that the 8-OHNVP glucuronide, 12-OHNVP glucuronide, 2-OHNVP glucuronide, and 3-OHNVP glucuronide possessed the same or approximately the same UV responses at 240 nm as the respective aglycon metabolites. When quantitated, 2-OHNVP glucuronide represented 23.3% of the metabolite pattern and 3-OHNVP glucuronide represented 35.2%. The 2-OHNVP glucuronide to 3-OHNVP glucuronide ratio was calculated to be 0.66 based on peak area.

MS and NMR Metabolite Identification. 14C-labeled 4-CANVP collected from male rat urine was analyzed by EI/CI direct probe MS. Two metabolites were observed in the collected fraction. The molecular weight of the unknown component 1 was 282 and the molecular weight of component 2 (4-CANVP) was 296 as determined by methane CI. The sample was analyzed by deutero-ammonia CI and the number of exchangeable hydrogens was determined. Each component in the mixture had two exchangeable hydrogens. The EI spectrum of 4-CANVP in rat urine was compared with the EI spectrum of authentic 4-CANVP as shown in Fig. 9. The EI spectrum of 4-CANVP is a mixture but all the fragment ions associated with 4-CANVP were observed in the spectrum. Table 4 shows the structure of 4-CANVP along with the structures of the major EI fragment ions. The molecular ion is observed at m/z 296 along with the (M-H)+ ion at m/z 295. Cleavage of methyl from the parent ion results in the fragment ion at m/z 281. Loss of COH from the parent ion resulted in the fragment ion at m/z 267. The loss of methyl followed by water results in the fragment ion at m/z 263. The fragment ion at m/z 164 is the result of the loss of C6H5NO from the parent with rearrangement to the structure shown in the table.

The 1H NMR analysis of radiolabeled 4-CANVP showed the presence of two components, as in the mass spectral analysis. The spectrum of the major component is consistent with a carboxylic acid substitution at the four position of NVP. A sample of authentic 4-CANVP, prepared by SPE, dried, and dissolved in dimethyl sulfoxide-d6, was analyzed by 1H NMR for comparison. The resultant spectrum is consistent with that of the major component of the 4-CANVP metabolite as shown in Fig. 10.

The biotransformation pathway of NVP in male and female mice, rats, rabbits (female only), dogs, cynomolgus monkeys, and chimpanzees is shown in Fig. 11. It is identical with the pathways observed in humans (Riska et al., 1999)

### Discussion

Parent compound was absent or present as a small percentage of the total radiochromatographic radioactivity in the urine of all animals except for a single male cynomolgus monkey that excreted 27% of the drug unchanged. Additionally, [14C]NVP accounted for little or none of the total fecal radioactivity except in the male and female dog. These results indicate that essentially the entire dose of NVP was absorbed in both sexes in all species except the male and female dog. Because preliminary data showed that [14C]NVP was barely detectable in bile from male dog after p.o. administration, the contribution

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<th>Fragment Ion (m/z)</th>
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</table>
of biliary recirculation to the quantity of parent compound in the fecal metabolite pattern can be considered minimal. Taken together, the data indicate incomplete absorption of the dose in the dog.

The comparative metabolite patterns in all the animals examined demonstrated that [14C]NVP undergoes extensive oxidative metabolism with subsequent glucuronidation of hydroxylated metabolites and excretion into the urine and feces. Glucuronidation was a major pathway in both sexes of all species. The five [14C]NVP oxidative metabolites identified as major and/or minor metabolites in urine, feces, bile (rat only), and/or plasma (rat and dog only) were 2-, 3-, 8-, and 12-OHNVP and 4-CANVP. 4-CANVP was the only identified oxidative product that appeared as a major urinary metabolite in both sexes in all species, except for the female rat. In this case, the three unidentified (M9/M10/M11) metabolites and 12-OHNVP were the major biotransformation products excreted in urine. Preliminary mass spectral data indicated that these three unidentified metabolites were formed by hydroxylation of NVP. Because comparison with authentic standards showed they are not hydroxylations in the 2, 3, 7, 8, or 9 position, we are led to think they may occur on some ring-opened structure. Confirmation of these structures has not yet been achieved. The major or minor identified glucuronides excreted in all animals were 2-, 3-, and 12-OHNVP conjugates. 8-OHNVP glucuronide appeared as a minor metabolite. Whereas only traces of glucuronide conjugates were excreted in rat urine, large quantities of 2-, 3-, and 12-OHNVP were excreted in bile. The major metabolite in rat plasma

![Image](image-url)

**Fig. 10.** 1H NMR spectral comparison of authentic 4-CANVP and the 4-CANVP rat urine metabolite, expanded aromatic region.

![Image](image-url)

**Fig. 11.** Biotransformation of [14C]NVP in mouse, rat, rabbit, dog, and monkey urine.
was 12-OHNVP, with the male having a greater amount than the female. This is consistent with the slower rate of metabolism, and consequent higher plasma concentrations of parent compound, in female rats. 3- and 12-OHNVP, 4-CANVP, and 3-, 8-, and 12-OHNVP glucuronide conjugates were all present in male and female beagle dog plasma as major and/or minor metabolites.

There were no gender differences in the metabolism of [14C]NVP in the mouse and beagle dog. In cynomolgus monkeys, the metabolism was qualitatively similar in the two sexes, but differences were observed in the amounts of the various metabolites produced. Metabolites excreted in rat urine and feces were qualitatively similar except that the male produced 4-CANVP as the sole major metabolite and the female formed M9, M10, and M11 complex. After i.v. administration, the male and female profiles were also qualitatively similar.

When the urine and fecal metabolite patterns are compared within each sex after p.o. and i.v. administration of NVP, it was apparent that the male and female profiles were also qualitatively similar except for the amounts of the various metabolites produced. Metabolite ratios are indicative of the renal oxidation of 12-OHNVP. The plasma major metabolite in the male rat. Finally, urinary 4-CANVP because 4-CANVP was not observed in plasma. 12-OHNVP was the major metabolite excreted via the bile into the feces or via the plasma into the gut by intestinal bacteria. The i.v. biliary metabolite profiles in the male and female rat clearly demonstrate this point. Biliary metabolite patterns with both sexes showed the presence of 2-, 8-, and 12-OHNVP glucuronides as major or minor metabolites. Male and female rat feces, as previously stated, lacked these metabolites. Additionally, the quantities of aglycones in feces were smaller than the corresponding biliary glucuronide conjugates. In the male rat, this result demonstrated that 12-OHNVP was absorbed and then oxidized by the liver to yield 4-CANVP, which was then excreted into the bile to accumulate in the feces. In vitro rat microsomal data substantiates the oxidation of 12-OHNVP to 4-CANVP (D. Erickson, personal communication). Also, the plasma metabolite patterns substantiate this result because 4-CANVP was not observed in plasma. 12-OHNVP was the plasma major metabolite in the rat. Finally, urinary 4-CANVP (73.6%) could only have resulted from the renal metabolism of plasma 12-OHNVP.

In the female rat, the aglycon metabolites (2-, 3-, and 12-OHNVP) produced by intestinal microflora appear to have been partially or totally absorbed and then oxidized further by the liver to yield the unidentified metabolites (M9, M10, or M11). Apparently these were then excreted via the bile into the feces or via the plasma into the gut, as gleaned from the 4-h p.o. plasma profiles and the 0- to 48-h fecal profiles. Like the male, the female rat plasma and urine metabolite ratios are indicative of the renal oxidation of 12-OHNVP. The biliary recirculation of [14C]NVP equivalents after intraduodenal administration has been demonstrated previously (Norris et al., 1992).

NVP was administered to chimpanzees in a pharmacokinetic study designed to provide data for allometric scaling to predict the human dosing regimen and pharmacokinetics. This gave a unique opportunity to collect urine for metabolite profiles. Female chimpanzee metabolite patterns were found to be qualitatively similar to those in male humans (Riska et al., 1996). In the 0- to 12-h metabolite urinary profiles in chimpanzees, the amount of unchanged drug ranged from 4 to 25% of the chromatographic peak area. Overall, the data indicate that, although the female chimpanzee may be the most relevant of the five laboratory species to the human as a toxicological, metabolic, and pharmacokinetic model for NVP, the toxicology species used (rat, dog, rabbit, and mouse) are good models as well.

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References


