Identification of Metabolites of Azaperone in Horse Urine[‖,⊥]

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Abstract □ Two metabolites of the tranquilizer azaperone were extracted from alkalinized horse urine after treatment with β-glucuronidase/sulfatase from limpets *(Patella vulgata*). The metabolites were identified by a combination of independent chemical synthesis and GC/MS and ^{1}H NMR analysis. The metabolites were identified as 1-(fluorophenyl)-4-[4-(5-hydroxy-2-pyridinyl)-1-piperazinyl]-1-butanol, designated as 5′-hydroxyazaperol, and 1-(fluorophenyl)-4-[4-(5-hydroxy-2-pyridinyl)-1-piperazinyl]-1-butanone, designated as 5′-hydroxyazaperone. A TLC screening test was developed for detecting both metabolites in basic extracts of horse urine treated with β-glucuronidase/sulfatase. The screening test was used to detect azaperone metabolites in extracts of horse urine collected for 24 h after intravenous administration of azaperone. The administration of azaperone to horses was confirmed by GC/MS identification of 5′-hydroxyazaperone and 5′-hydroxyazaperol from basic extracts of horse urine treated with β-glucuronidase/sulfatase. The extracted metabolites were treated with bis(trimethylsilyl)acetamide to produce trimethylsilyl (TMS) ether derivatives, and mass spectra and retention times were compared to those of the synthesized metabolites treated in the same manner.

Azaperone (1-(4-fluorophenyl)-4-[4-(2-pyridinyl)-1-piperazinyl]-1-butanone) (**1**) is a butyrophenone tranquilizer approved for use in swine to prevent stress-related injuries, loss of weight, and mortality during transport. There have been recent reports of azaperone use in performance horses.[1,2] The rationale for administration of azaperone to horses is not clear since it has been reported to produce tranquilization after intramuscular doses of 0.40−0.80 mg/kg of body weight[3] but excitement and ataxia after intravenous doses of 0.29−0.57 mg/kg of body weight.[4] The administration of drugs such as azaperone to performance horses is prohibited by racing commission and horse show association rules. However, regulatory action requires unequivocal identification of the drug or its metabolites from test samples.

Reduction of the carbonyl group in azaperone (**1**) to yield azaperol (**2**) in Figure 1 is a major metabolic route for azaperone in swine.[5] Oxidative removal of the pyridyl group resulting in 1-(4-fluorophenyl)-4-(1-piperazinyl)-1-butanone (**3**) and acetylation of this metabolite on the free piperazine nitrogen resulting in 1-(4-fluorophenyl)-4-(4-acetyl-1-piperazinyl)-1-butanone (**4**) are the major metabolic pathways in the rat.[6] Analysis of urine samples collected from experimental horses administered azaperone at various laboratories has indicated that azaperone is not present at significant concentrations and other known azaperone metabolites, including azaperol, are not the major metabolites in horse urine.[3,4] Recently several hydroxylated metabolites of azaperone were identified by LC/MS/MS from extracts of horse urine after treatment with β-glucuronidase.[7] However, these metabolites were incompletely characterized because the position of hydroxylation was not determined. Therefore, the purposes of this study were to identify hydroxylated metabolites of azaperone in extracts of horse urine, to determine the position of hydroxylation in these metabolites, and to develop effective TLC and GC/MS methods to detect and confirm the identities of azaperone metabolites in horse urine.

Experimental Section

Chemicals—Azaperone hydrochloride injectable solution (Stresnil) was purchased from Pitman-Moore, Inc. (Washington Crossing, NJ); D-saccharic acid 1,4-lactone monohydrate and deuterated dichloromethane (99.6% isotopic purity) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Type L-II β-glucuronidase (containing sulfatase activity) from limpets (*Patella vulgata*) was purchased from Sigma Chemical Co. (St. Louis, MO). Analytical TLC plates (silica gel 60 F_{254}, 5 × 10 cm, 0.25 mm thickness) were purchased from EM Science, Inc. (Cherry Hill, NJ). Preparative TLC plates (silica gel 60 F_{254}, 20 × 20 cm, 0.5 mm thickness) were purchased from Analtech (Newark, DE).

Dragendorff reagent solution was prepared by combining 10-mL of bismuth subnitrate:glacial acetic acid:water (2:25:100, w/v/v), 10-mL of 40% aqueous potassium iodide, 20-mL of glacial acetic acid, and 100-mL of water. Folin-Denis reagent was prepared by refluxing 10 g of sodium tungstate, 2 g of 12-molybdosilic acid, 5-mL of concentrated phosphoric acid, and 50-mL of water for 2 h; the cooled mixture was diluted to 100-mL with water.

Administration of Azaperone to Test Horses—Five healthy mares weighing between 478 and 565 kg were used in this study. The horses ranged in age from 3 to 10 years and were either Thoroughbred or Standardbred breeds. All horses were in good health as determined by physical examination and hemogram. The horses were maintained in open pasture with free access to feed and water before the experimental trial. The horses were placed in box stalls and feed was withheld for 2 h before and 8 h after drug administration. Water was available *ad libitum*.

A balloon-tipped catheter was placed in the bladder of each horse for urine sample collection. Azaperone, as azaperone hydrochloride aqueous solution (40 mg/mL, Stresnil, Pitman-Moore, Inc.), was administered iv to each horse via the right jugular vein at a total dose of 40 mg/horse (0.071−0.084 mg/kg body weight). Urine samples were collected before and from 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 6, 6 to 8, and at 24 h after drug administration. Urine samples were immediately frozen and stored at −20 °C until laboratory analysis.

Isolation and Identification of Azaperone Metabolites from Horse Urine—Urine samples were extracted under the following conditions and the resulting isolates were subjected to analytical TLC.

Extraction of Alkalinized Urine—a 9-mL urine aliquot from each collection interval was alkalinized with 2-mL of 1.0 M sodium carbonate solution containing bromothymol blue (200 mg/L). The mixtures were then extracted with 5-mL of dichloromethane:2-propanol (3:1, v/v) by end-over-end rotation at 20 rpm for 5 min. The phases were separated by centrifugation at 1000*g* for 5 min and the aqueous phases were discarded. The organic phases were transferred

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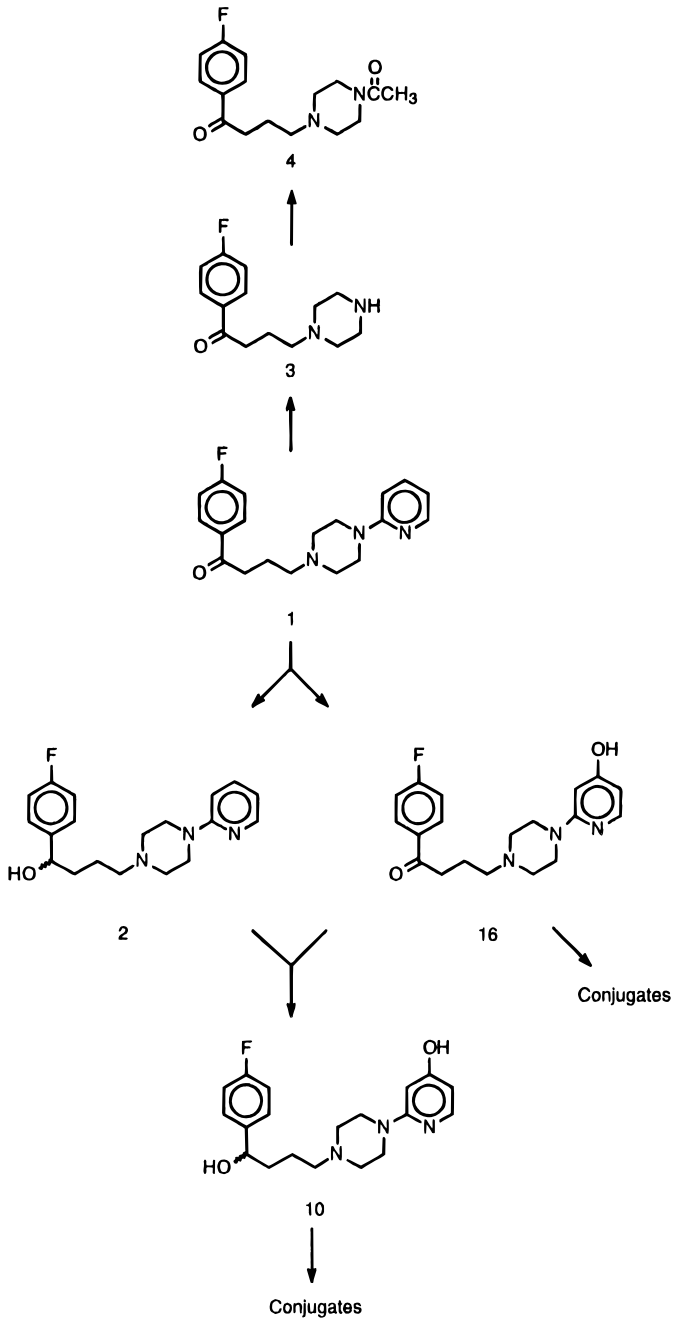

Figure 1—Metabolism of azaperone in pigs, rats, and horses.

to clean 5-mL conical centrifuge tubes and evaporated under a flow of nitrogen in a water bath at 40−45 °C.

Extraction of pH-Neutral Urine—a 5-mL urine aliquot from each collection interval was neutralized with 2-mL of 0.67 M phosphate buffer (pH 6.0). The mixtures were then extracted with 5-mL of dichloromethane:2-propanol (3:1, v/v) by end-over-end rotation at 20 rpm for 5 min. The phases were separated and the organic phases concentrated as described above.

Extraction of Acidified Urine—a 5-mL urine aliquot from each collection interval was acidified with 5-mL of a saturated aqueous solution of KH_2PO_4 (adjusted to pH 3.3 with 6 N hydrochloric acid). The mixtures were then extracted with 5-mL of dichloromethane: petroleum ether (10:1, v/v) by end-over-end rotation at 20 rpm for 5 min. The phases were separated and the organic phases were concentrated as described above.

Glucuronidase/Sulfatase Hydrolysis and Extraction of Alkalinized Urine Samples—A 5-mL urine aliquot from each collection interval was acidified with 2-mL of 1.0 M acetate buffer (pH 5.0) and 1-mL of

Table 1—Thin layer Chromatographic R_f Values for Azaperone, Azaperol, 5′-Hydroxyazaperol (10), and 5′-Hydroxyazaperone (16) on Merck Precoated Silica Gel 60 F-254 TLC Plates (0.25 mm Thickness) with Solvent Systems A–G[a]

	R_f						
Compound	A	B	C	D	E	F	G
Azaperone	0.88	0.90	0.58	0.24	0.00	0.68	0.08
Azaperol	0.90	0.74	0.42	0.12	0.00	0.48	0.04
Isolated **10**	0.90	0.40	0.06	0.00	0.00	0.22	0.08
Synthetic **10**	0.90	0.40	0.06	0.00	0.00	0.22	0.08
Isolated **16**	0.91	0.52	0.12	0.00	0.00	0.40	0.14
Synthetic **16**	0.91	0.53	0.12	0.00	0.00	0.40	0.14

[a] Solvent systems: A, methanol:ammonium hydroxide solution (100:1.5, v/v); B, ethyl acetate:methanol:ammonium hydroxide solution (80:15:5, v/v); C, chloroform:ethanol (9:1, v/v); D, chloroform:ethanol (19:1, v/v); E, chloroform: cyclohexane:glacial acetic acid (2:2:1, v/v); F, chloroform:methanol:propionic acid (72:18:10, v/v); G, ethyl acetate:methanol:glacial acetic acid (8:1:1, v/v).

β-glucuronidase/sulfatase reagent (5000 units of β-glucuronidase/mL in distilled water) was added. The contents of each tube were vortex-mixed and then heated at 65 °C for 4 h.[8]

The mixtures were cooled to room temperature, and 0.5-mL of 10% ascorbic acid in water was added to each tube. The pH of each mixture was adjusted to 8.5−9.2 with 6 N hydrochloric acid or concentrated ammonium hydroxide:water (1:1, v/v) as needed. The mixtures were extracted with 5-mL of dichloromethane:2-propanol (10:1, v/v) by end-over-end rotation at 20 rpm for 5 min and centrifuged at 1000*g* for 5 min. The aqueous phases were discarded and the organic phases were transferred to clean tubes. The organic phases were then extracted with 3.0-mL of 1 N sulfuric acid, the organic phases were discarded, and the aqueous phases were transferred to clean tubes.

After addition of 0.2-mL of 10% ascorbic acid to each tube, the pH was adjusted to 8.5−9.2 with concentrated ammonium hydroxide solution. Then, 5-mL of dichloromethane:2-propanol (10:1, v/v) was added to each tube and the contents extracted as above.

Verification of Glucuronic Acid Conjugation by Glucuronidase and Sulfatase Inhibition—The presence of glucuronic acid conjugates of azaperone metabolites was demonstrated by inhibiting β-glucuronidase activity by the addition of the competitive inhibitor D-saccharic acid 1,4-lactone monohydrate and by inhibiting sulfatase activity by substitution of phosphate buffer for acetate buffer in separate experiments.[9]

Three 5-mL aliquots of the 0 to 1 h urine samples were acidified by the addition of 2-mL of 1 M sodium acetate buffer (pH 5.0) and mixed with 1-mL of β-glucuronidase/sulfatase reagent (5000 units of β-glucuronidase/mL). Then 0.1-mL of water was added to the first aliquot, 0.1-mL of 8.1 mM D-saccharic acid 1,4-lactone monohydrate to the second aliquot, and 0.1-mL of 81 mM D-saccharic acid 1,4-lactone monohydrate to the third aliquot. The mixtures were incubated at 40 °C for 3 h and extracted as described above.

Sulfatase activity in the β-glucuronidase/sulfatase reagent was inhibited by substituting 0.4 M phosphate buffer for 1 M acetate buffer.[9] A 5-mL aliquot of the 0 to 1 h urine sample was acidified with 2-mL of 0.4 M KH_2PO_4 buffer (pH 5.0) and mixed with 1-mL of β-glucuronidase/sulfatase reagent (5000 units of β-glucuronidase/mL). A positive control sample was acidified with 1 M acetate buffer and processed with the test sample. These mixtures were incubated at 40 °C for 3 h and then extracted as described above.

Thin Layer Chromatographic Analysis of Urine Extracts—Residues obtained from urine extracts were analyzed by TLC using Merck precoated silica gel 60 F-254 plates (0.25 mm thickness). Each residue was dissolved in 20 μL of ethyl acetate and 1 μL was spotted on the origin of each of seven TLC plates. Each TLC plate was developed a distance of 5.0 cm in one of the solvent developing systems described in Table 1.

The locations of azaperone metabolites were visualized by their blue fluorescence under 350-nm UV light, absorption under 254-nm UV light, and color formation after sequential application of Folin-Dennis spray reagent, followed by exposure to ammonia fumes, and Dragendorff spray reagent, followed by 5% sodium nitrite solution.

Preparative Isolation of Metabolites for Structural Analysis—Preparative isolation of azaperone metabolites extracted

from the 0−1 h urine samples was performed using the β-glucuronidase/sulfatase hydrolysis method described above. The hydrolysis was carried out on 200-mL of urine in 40 5-mL urine aliquots. The final organic extracts from the 40 aliquots were combined in a 500-mL boiling flask and evaporated at 45 °C in a flash evaporator. Residual water in the sample was evaporated azeotropically with acetonitrile under a stream of nitrogen.

The residue was dissolved in 400 μL of ethyl acetate and subjected to preparative TLC on precleaned 20 cm × 20 cm silica gel GF preparative TLC plates (0.5 mm thickness, Analtech). The TLC plates were precleaned by developing them twice in HPLC-grade methanol and twice in HPLC-grade acetonitrile before use. The extract was streaked along the origin, and the TLC plate was developed 10 cm in solvent system F. Two separate metabolite zones were visualized under 350-nm and 254-nm UV light. The two metabolite zones were separately collected with Kontes 3-mL zone collectors and the metabolites were eluted from the silica gel with 3−4-mL of HPLC-grade methanol. The methanol eluates were concentrated under nitrogen on a water bath at 45 °C.

The isolated metabolites were each dissolved in 150 μL of ethyl acetate:methanol (2:1, v/v) and rechromatographed in solvent system F. The metabolite zones were visualized and collected as before. The TLC isolation procedure was repeated using solvent system C and then using solvent system B. The isolated metabolites were each dissolved in 2.5-mL of a saturated aqueous solution of sodium borate and extracted with 2.5-mL of dichloromethane by vortex-mixing for 30 s and then centrifuging to separate the layers. The dichloromethane solution was collected and the aqueous phase was extracted again with dichloromethane. The extracts were combined and concentrated under a flow of nitrogen. The isolated metabolites were then dried in a vacuum desiccator over phosphorous pentoxide.

Trimethylsilylation of Metabolites for GC/MS Analysis—The two isolated metabolites and synthesized metabolites were mixed separately with 20 μL of N,O-bis(trimethylsilyl)acetamide (BSTFA; Pierce, Rockford, IL) in capped, conical tubes and heated for 30 min at 65 °C. The mixtures were diluted with 20 μL of ethyl acetate before GC/MS analysis.

Low-Resolution GC/MS Analysis of Metabolites Isolated from Urine Extracts—The GC/MS instrument was a Hewlett-Packard model 5970 mass selective detector and model 5890 gas chromatograph with splitless injector, capillary direct interface, and autosampler. Aliquots (2 μL) of the BSTFA-treated sample extracts and synthesized metabolites were injected via the splitless injector at 280 °C on to a 15 m × 0.251 mm i.d. DB-1 capillary column (0.25 μm film thickness, J & W Scientific, Rancho Cordova, CA) at an initial column oven temperature of 150 °C. After 1.0 min, the temperature of the column oven was increased at a rate of 20 °C/min to a final temperature of 280 °C and was maintained at that temperature for 12.5 min. Helium was used as the carrier gas at a flow rate of 1-mL/min. The mass spectrometer was operated under electron-impact ionization conditions at 70 eV. Perfluorotributylamine was used as the reference compound.

High-Resolution GC/MS Analysis—High-resolution GC/MS analysis of isolated metabolites was done at the Ohio State University Physical Chemistry Instrument Center using a VG model 70−250S double-focusing magnetic sector instrument and a Hewlett-Packard model 5890 gas chromatograph. Aliquots of the BSTFA-treated sample extracts were injected via the splitless injector at 280 °C onto a 30 m × 0.251 mm i.d. capillary column (0.25 μm film thickness, DB-1, J & W Scientific) at an initial column oven temperature of 100 °C. After 3.0 min, the column oven temperature was increased at a rate of 15 °C/min to a final temperature of 280 °C. Helium was used as the carrier gas at a flow rate of 1 mL/min. All spectra were obtained under electron-impact ionization conditions at 70 eV and a source temperature of 200 °C. Perfluorokerosene was used as the reference compound.

Nuclear Magnetic Resonance Spectrometry—The purified metabolites were dissolved in 1−2-mL of HPLC-grade ethyl acetate and transferred to 5 × 250 mm NMR tubes (Wilmad, Buena, NJ). The ethyl acetate was concentrated to dryness under nitrogen at room temperature, and the tubes were then placed in a vacuum desiccator containing phosphorus pentoxide for 2 days before ^{1}H NMR analysis. The residues in the NMR tubes were dissolved in CD_2Cl_2 for ^{1}H NMR analysis with a Bruker AM-500 system.

Synthesis of Intermediates and Metabolites—Infrared spectra were recorded on a Perkin-Elmer model 357 spectrometer. Nuclear magnetic resonance spectra were determined on a Varian 400 MHz or Gemini 200 MHz NMR spectrometer. Chemical shifts are reported as parts per million relative to tetramethylsilane as an internal standard. Electron-impact ionization mass spectra of synthetic compounds were determined at an electron energy of 70 eV with a Kratos Concept 1H spectrometer. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA. Column chromatography using Machery Nagel silica gel is referred to as "chromatographed on silica gel".

1-(4-Fluorophenyl)-4-[4-(2-pyridinyl)-1-piperazinyl]-1-butanol (Azaperol) (2)—To a solution of 1.31 g (4 mmol, 1 equiv) of azaperone free base (**1**) in 30-mL of anhydrous ether and 10-mL of anhydrous tetrahydrofuran at 0 °C under a nitrogen atmosphere was slowly added a solution of 304 mg (8 mmol, 8 equiv) of lithium aluminum hydride in 10-mL of ether. The mixture was stirred at 25 °C for 30 min and carefully hydrolyzed by adding 0.5-mL of water dropwise with stirring over a 10 min period. The precipitate was collected and washed thoroughly with ether. The filtrate was washed with water and brine, dried over anhydrous magnesium sulfate, and concentrated to afford 1.21 g (92%) of azaperol (**2**): IR (KBr) 3100 (br), 2838, 1594, 1564 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.60−2.00 (m, 4, aliphatic CH_2), 2.43−2.75 (m, 6, aliphatic and piperazine NCH_2), 3.60 (t, J = 4.7 Hz, 4, piperazine NCH_2), 4.60−4.70 (m, 1, CHOH), 6.63 (m, 2, pyridine H-3 and H-5), 6.95−7.04 (m, 2, ArH), 7.30−7.40 (m, 2, ArH), 7.42−7.52 (m, 1, pyridine H-4), 8.18 (d, J = 5.5 Hz, 1, pyridine H-6); ^{13}C NMR ($CDCl_3$) δ 23.8, 39.9, 45.0, 53.1, 59.1, 73.3, 107.5, 114.0, 115.1, 115.6, 127.6, 127.8, 138.1, 142.0, 142.0, 148.5, 159.9, 160.0, 164.8; exact mass spectrum calculated for $C_{19}H_{24}FN_3O$ 329.1905, found 329.1903.

1-(4-Fluorophenyl)-4-[4-(2-pyridinyl)-1-piperazinyl]-1-butyl Acetate (5)—To a solution of 1.13 g (3.42 mmol, 1 equiv) of **2** in 5-mL of anhydrous pyridine was added 698 mg (645 μL, 6.84 mmol, 2 equiv) of acetic anhydride followed by 50 mg of 4-(*N*,*N*-dimethylamino)-pyridine at 0 °C. The mixture was stirred at 0 °C for 2 h and at 25 °C for 18 h. The solvent was removed under reduced pressure, and the residue was dissolved in water. The solution was diluted with a saturated solution of sodium bicarbonate and extracted with chloroform. The combined chloroform solutions were washed with water and brine, dried over anhydrous magnesium sulfate, and concentrated to afford 1.22 g (96%) of **5**: IR (TF) 1737, 1594 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.35−1.70 (m, 2, aliphatic CH_2), 1.70−2.00 (m, 2, aliphatic CH_2), 2.06 (s, 3, $OCOCH_3$), 2.37 (t, J = 7.4 Hz, 2, NCH_2CH_2), 2.50 (t, J = 5 Hz, 4, piperazine CH2), 3.53 (t, J = 5.0 Hz, 4, piperazine CH_2), 5.74 (t, J = 6.9 Hz, 1, CHOH), 6.55−6.65 (m, 2, pyridine H-3 and H-5), 7.02 (t, J = 8.7 Hz, 2, ArH), 7.25−7.40 (m, 2, ArH), 7.42−7.50 (m, 1, pyridine H-4), 8.15−8.22 (m, 1, pyridine H-6); ^{13}C NMR ($CDCl_3$) δ 21.4, 23.0, 34.3, 45.4, 53.3, 58.4, 75.5, 107.5, 113.7, 115.6, 116.0, 128.7, 128.9, 136.9, 136.9, 138.0, 148.5, 160.1, 160.5, 165.4, 171.0. *Anal.*—Calculated for $C_{21}H_{26}FN_3O_2$: C, 67.90, H, 7.06. Found: C, 67.98; H, 7.08.

1-(4-Fluorophenyl)-4-[4-(5-bromo-2-pyridinyl)-1-piperazinyl]-1-butanol (6)—To a stirred solution of 333 mg (1.02 mmol, 1 equiv) of **2** in 10-mL of acetic acid at 25 °C was slowly added a solution of 163 mg (1.02 mmol, 1 equiv) of bromine in 2-mL of acetic acid. The mixture was stirred for 30 min. The solvent was removed under reduced pressure. The residue was dissolved in water and treated with a saturated solution of sodium bicarbonate until basic and extracted with three 20-mL portions of chloroform. The combined chloroform solutions were washed successively with sodium bicarbonate solution and brine, dried over anhydrous magnesium sulfate, and concentrated to afford 410 mg (99%) of **6**: IR (KBr) 3349, 2938, 2839, 1605, 1585, 1548 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.60−2.02 (m, 4, aliphatic CH_2), 2.40−2.75 (m, 6, aliphatic NCH_2 and piperazine CH_2), 3.58 (t, J = 5 Hz, 4, piperazine CH_2), 4.60−4.70 (m, 1, CHOH), 6.53 (d, J = 9 Hz, 1, pyridine H-3), 7.00 (t, J = 9 Hz, 2, ArH), 7.30−7.37 (m, 2, ArH), 7.53 (dd, J = 9, 2.5 Hz, 1, pyridine H-4), 8.19 (d, J = 2.5 Hz, 1, pyridine H-6); ^{13}C NMR ($CDCl_3$) δ 23.1, 39.9, 45.0, 49.1, 52.1, 53.0, 60.1, 73.4, 108.3, 108.8, 115.2, 115.6, 127.6, 127.8, 140.3, 141.9, 142.0, 149.1, 158.4, 160.0, 164.8.

1-(4-Fluorophenyl-4-[4-(5-bromo-2-pyridinyl)-1-piperazinyl]-1-butyl Acetate (7)—To a stirred solution of 1.20 g (3.23 mmol, 1 equiv) of **5** in 25-mL of acetic acid at 25 °C was slowly added a solution of 517 mg (3.23 mmol, 1 equiv) of bromine in 25-mL of acetic acid. The mixture was stirred for 15 min and concentrated under reduced pressure. The residue was diluted with water, made basic by addition of a saturated solution of sodium bicarbonate, and extracted with

chloroform. The combined chloroform solutions were washed with water and brine, dried over anhydrous magnesium sulfate, and concentrated to afford 1.45 g (100%) of **7**: IR (KBr) 1733, 1604, 1584, 1547, 1510 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.40−1.65 (m, 2, aliphatic CH_2), 1.74−2.00 (m, 2, aliphatic CH_2), 2.07 (s, 3, $OCOCH_3$), 2.38 (t, J = 7.4 Hz, 2, NCH_2CH_2), 2.49 (t, J = 5 Hz, 4, piperazine CH_2), 3.50 (t, J = 5 Hz, 4, piperazine CH_2), 5.74 (t, J = 6.9 Hz, 1, CHOAc), 6.53 (d, J = 9 Hz, 1, pyridine H-3), 6.98−7.07 (m, 2, ArH), 7.29−7.35 (m, 2, ArH), 7.52 (dd, J = 9, 2.5 Hz, 1, pyridine H-4), 8.18 (d, J = 2.5 Hz, 1, pyridine H-6); ^{13}C NMR ($CDCl_3$) δ 21.4, 22.9 34.2, 45.3, 53.0, 58.3, 75.5, 108.1, 108.8, 115.6, 116.1, 128.7, 128.9, 136.8, 136.9, 140.2, 149.0, 158.5, 160.5, 165.4, 171.0; exact mass spectrum calculated for $C_{21}H_{25}BrFN_3O_2$ 449.1116, found 449.1115.

1-(4-Fluorophenyl)-4-[4-(5-bromo-2-pyridinyl)-1-piperazinyl]-1-(*tert*-butyldimethylsiloxy)butane (8)—To a solution of 408 mg (1 mmol, 1 equiv) of **6** in 2-mL of anhydrous *N,N*-dimethylformamide (DMF) was added 170 mg (2.5 mmol, 2.5 equiv) of imidazole. The mixture was stirred at 25 °C for 5 min, and 226 mg (1.5 mmol, 1.5 equiv) of *tert*-butyldimethylsilyl chloride was added. The solution was stirred at 25 °C for 18 h. The mixture was diluted with ethyl acetate and washed thoroughly with water. The combined aqueous phase was extracted once with ethyl acetate, and the combined ethyl acetate solutions were washed with brine, dried over anhydrous magnesium sulfate, concentrated, and chromatographed on silica gel using ethyl acetate:hexane (1:1, v/v) to afford 450 mg (86%) of **8**: IR (TF) 2952, 2885, 2856, 1605, 1584, 1548, 1509 cm^{-1}; ^1H NMR ($CDCl_3$) δ −0.15 (s, 3, CH_3Si), 0.03 (s, 3, $SiCH_3$), 0.88 (s, 9, $SiC(CH_3)_3$), 1.40−1.75 (m, 4, aliphatic CH_2), 2.34 (t, J = 7.2 Hz, 2, aliphatic NCH_2), 2.48 (t, J = 5.1 Hz, 4, piperazine CH_2), 3.49 (t, J = 5.1 Hz, 4, piperazine CH_2), 4.66 (t, J = 6.3 Hz, 1, CHOTBS), 6.53 (d, J = 9 Hz, 1, pyridine H-3), 6.90−7.03 (m, 2, ArH), 7.22−7.29 (m, 2, ArH), 7.52 (dd, J = 9, 2.5 Hz, 1, pyridine H-4), 8.18 (d, J = 2.5 Hz, 1, pyridine H-6); ^{13}C NMR ($CDCl_3$) δ −4.87, −4.59, 18.3, 22.7, 25.9, 38.9, 45.4, 53.1, 58.8, 74.5, 108.0, 108.8, 115.1, 115.5, 127.7, 127.9, 140.2, 141.8, 141.8, 149.0, 158.6, 159.0, 164.8. *Anal.*—Calculated for $C_{25}H_{37}BrFN_3SiO$: C, 57.46, H, 7.14. Found: C, 57.42; H, 7.16.

1-(5-Nitro-2-pyridinyl)piperazine (12)—To a solution of 860 mg (10 mmol, 2 equiv) of piperazine (**11**) and 1.02 g (5 mmol, 1 equiv) of 2-bromo-5-nitropyridine in 5-mL of isoamyl alcohol was added 530 mg (5 mmol, 1 equiv) of anhydrous sodium carbonate. The mixture was refluxed under a Dean Stark trap for 2 h. The mixture was cooled, the solids were removed by filtration, and the clear filtrate was concentrated and chromatographed on silica gel using methanol: chloroform (1:5, v/v) to afford 880 mg (85%) of **12**: IR (KBr) 3346, 2953, 2900, 2834, 1607, 1568, 1525 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.78 (s, 1, NH), 2.99 (t, J = 5.1 Hz, 4, piperazine CH_2), 3.75 (t, J = 5.1 Hz, 4, piperazine CH_2), 6.57 (d, J = 9.5 Hz, 1, pyridine H-3), 8.20 (dd, J = 9.5, 2.7 Hz, 1, pyridine H-4), 9.04 (d, J = 2.7 Hz, 1, pyridine H-6).

1-(4-Fluorophenyl)-4-[4-(5-nitro-2-pyridinyl)-1-piperazinyl]-1-butanone (13) from 12—To a solution of 416 mg (2 mmol, 1 equiv) of **12** and 8.02 mg (4 mmol, 2 equiv) of 4-chloro-1-(4-fluorophenyl)-1-butanone in 10-mL of *n*-butanol was added 212 mg (2 mmol, 1 equiv) of anhydrous sodium carbonate and 150 mg (1 mmol, 0.5 equiv) of sodium iodide. The mixture was refluxed under a nitrogen atmosphere for 20 h, and the solvent was removed by distillation under reduced pressure. The residue was diluted with chloroform, washed with water, sodium bicarbonate solution and brine, dried over magnesium sulfate, concentrated, and chromatographed on silica gel using methanol:chloroform (1:19, v/v) to afford 180 mg (24%) of **13**: IR (KBr) 2949, 1689, 1595, 1510 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.95−2.05 (m, 2, aliphatic CH_2), 2.40−2.60 (m, 6, NCH_2), 3.03 (t, J = 6.9 Hz, 1, $ArCOCH_2$), 3.71 (t, J = 4.6 Hz, 4, piperazine NCH_2), 6.56 (d, J = 9.5 Hz, 1, pyridine H-3), 7.14 (t, J = 8.5 Hz, 2, ArH), 7.99−8.10 (m, 2, ArH), 8.18 (dd, J = 9.5, 2.7 Hz, 1, pyridine H-4), 9.01 (d, J = 2.7 Hz, 1, pyridine H-6); ^{13}C NMR ($CDCl_3$) δ 21.5, 36.2, 45.0, 52.9, 57.8, 105.0, 116.0, 116.4, 131.1, 131.2, 133.5, 134.1, 135.3, 147.1, 160.9, 163.7, 199.1. *Anal.*—Calculated for $C_{19}H_{21}FN_4O_3$: C, 61.28, H, 5.68. Found: C, 61.33; H, 5.68.

1-(4-Fluorophenyl)-4-(1-piperazinyl)-1-butanone (3)—To a solution of 2.0 g (10 mmol, 1 equiv) of 4-chloro-1-(4-fluorophenyl)-1-butanone and 1.72 g (20 mmol, 2 equiv) of piperazine (**11**) in 10-mL of isoamyl alcohol was added 1.06 g (10 mmol, 1 equiv) of anhydrous sodium carbonate. The mixture was refluxed under a Dean Stark trap for 3 h. The solution was concentrated. The residue was dissolved in chloroform, washed with water, sodium bicarbonate solution and brine, dried over anhydrous magnesium sulfate, concentrated, and chromatographed on silica gel using ammonium hydroxide solution:methanol:chloroform (1:8:40, v/v) to afford 1.78 g (71%) of **3**: IR (KBr) 3435, 2954, 2823, 1677, 1600, 1507 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.85−2.02 (m, 2, aliphatic CH_2), 2.35−2.60 (m, 6, NCH_2), 2.80−3.05 (m, 6, $COCH_2$ and NCH_2), 3.25−3.36 (m, 1, NH), 7.05−7.20 (m, 2, ArH), 7.95−8.10 (m, 2, ArH).

1-(4-Fluorophenyl)-4-[4-(5-nitro-2-pyridinyl)-1-piperazinyl]-1-butanone (13) from 3—To a solution of 880 mg (3.52 mmol, 1 equiv) of **3** and 715 mg (3.52 mmol, 1 equiv) of 2-bromo-5-nitropyridine in 5-mL of isoamyl alcohol was added 373 mg (3.52 mmol, 1 equiv) of anhydrous sodium carbonate. The mixture was refluxed, with continuous removal of water with a Dean Stark trap, for 3 h. The solvent was removed by distillation under reduced pressure. The residue was dissolved in chloroform, washed with water, sodium bicarbonate solution and brine, dried over anhydrous magnesium sulfate, concentrated, and chromatographed on a silica gel column using methanol:chloroform (1:19, v/v) to afford 772 mg (59%) of **13**.

1-(4-Fluorophenyl)-4-[4-(5-hydroxy-2-pyridinyl)-1-piperazinyl]-1-butanone (16)—To a solution of 372 mg (1 mmol, 1 equiv) of **13** in 30-mL of methanol was added 50 mg of 10% palladium on carbon. The mixture was stirred under 50 psi of hydrogen at 25 °C for 6 h. The catalyst was removed by filtration through Celite, and the filtrate was concentrated to afford the crude amine (**14**). The amine (**14**) was dissolved in 10-mL of water containing 0.5-mL of concentrated sulfuric acid. The mixture was cooled to 0 °C; 83 mg (1.2 mmol, 1.2 equiv) of sodium nitrite in 1-mL of water was added dropwise; and stirring was continued at 0−5 °C for 15 min. The solution of the diazonium salt (**15**) was slowly added to a refluxing solution of 10-mL of concentrated sulfuric acid and 1.5 g of anhydrous sodium sulfate in 10-mL of water. The mixture was refluxed for 3 h. The solution was cooled, and the acid was neutralized first by adding a 20% sodium hydroxide solution and subsequently by adding sodium bicarbonate. The mixture was extracted with chloroform. The combined chloroform solutions were washed with sodium bicarbonate solution and brine, dried over anhydrous magnesium sulfate, concentrated, and chromatographed on a silica gel column using methanol:chloroform (1:5, v/v) to afford 80 mg (23%) of **16**: IR (KBr) 3540 (br), 2835, 1680, 1590 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.90−2.10 (m, 2, aliphatic CH_2), 2.48 (t, J = 7.2 Hz, 2, aliphatic NCH_2), 2.59 (br s, 4, piperazine CH_2), 3.00 (t, J = 6.9 Hz, 2, $COCH_2$), 3.34 (br s, 4, piperazine CH_2), 6.57 (d, J = 9.2 Hz, 1, pyridine H-3), 7.00−7.20 (m, 3, pyridine H-4 and ArH), 7.82 (d, J = 2.9 Hz, 1, pyridine H-6), 7.90−8.20 (m, 2, ArH); ^{13}C NMR ($CDCl_3$) δ 21.2, 36.3, 46.7, 53.1, 57.9, 109.6, 115.9, 116.3, 127.2, 131.1, 131.3, 135.2, 147.2, 155.0, 163.8. 168.9, 199.2.

1-(4-Fluorophenyl)-4-[4-(5-acetoxy-2-pyridinyl)-1-piperazinyl]-1-butanone (17)—To a solution of 26 mg (0.076 mmol, 1 equiv) of **16** in 0.5-mL of pyridine was added 15.5 mg (15 μL, 0.152 mmol, 2 equiv) of acetic anhydride and 2 mg (0.015 mmol, 0.2 equiv) of 4-(*N,N*-dimethylamino)pyridine. The mixture was stirred at 25 °C for 18 h. The solvent was removed under reduced pressure. The residue was dissolved in chloroform, washed with a saturated solution of sodium bicarbonate and brine, dried over anhydrous sodium bicarbonate, concentrated, and chromatographed on silica gel using methanol: chloroform (1:10, v/v) to afford 22 mg (76%) of **17**: IR (KBr) 1750, 1680, 1595 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.90−2.10 (m, 2, aliphatic H), 2.29 (s, 3, $OCOCH_3$), 2.47 (t, J = 7 Hz, 2, aliphatic NCH_2), 2.55 (t, J = 5 Hz, 4, piperazine CH_2), 3.02 (t, J = 7 Hz, 2, $COCH_2$), 3.50 (t, J = 5 Hz, 4, piperazine CH_2), 6.63 (J = 9.2 Hz, 1, pyridine H-3), 7.14 (t, J = 8.5 Hz, 2, ArH), 7.27 (dd, J = 9.2, 2.5 Hz, 1, pyridine H-4), 7.95−8.10 (m, 3, ArH and pyridine H-6). *Anal.*—Calculated for $C_{21}H_{24}FN_3O_2$: C, 65.44, H, 6.28. Found: C, 65.22; H, 6.33.

1-(4-Fluorophenyl)-4-[4-(5-hydroxy-2-pyridinyl)-1-piperazinyl]-1-butanol (10) from 17—To a solution of 20 mg (0.05 mmol, 1 equiv) of **17** in 3-mL of ether:tetrahydrofuran (2:1, v/v) at 0 °C was slowly added a solution of 8 mg (0.20 mmol, 8 equiv) of lithium aluminum hydride in 1-mL of ether. The mixture was stirred at 25 °C for 30 min and carefully hydrolyzed by adding a drop of water. The precipitate was removed by filtration and washed thoroughly with ether. The combined filtrate was washed with water and brine, dried over anhydrous magnesium sulfate, concentrated, and chromatographed on silica gel using methanol:chloroform (1:5, v/v) to afford 17 mg (94%) of **10**: IR (KBr) 3432, 3104, 2937, 1605, 1577, 1498 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.70−2.10 (m, 4, aliphatic CH_2), 2.45−2.90 (m, 6, aliphatic and piperazine CH_2), 3.20−3.40 (m, 4, piperazine CH_2), 4.65−4.75 (m, 1, CHOH), 6.34 (d, J = 8.8 Hz, 1, pyridine H-3), 6.92−

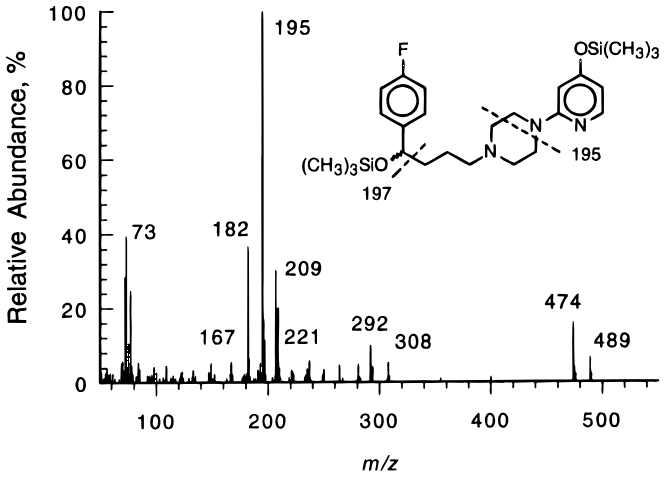

Figure 2—Electron-impact ionization mass spectrum of the TMS ether derivative of 5′-hydroxyazaperol (**10**) isolated from horse urine.

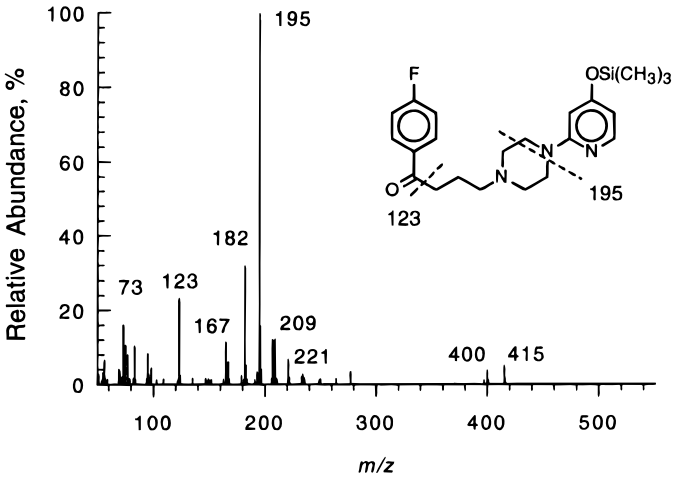

Figure 3—Electron-impact ionization mass spectrum of the trimethylsilyl derivative of 5′-hydroxyazaperone (**16**) isolated from horse urine.

7.10 (m, 3, ArH and pyridine H-4), 7.30−7.40 (m, 2, ArH), 7.85 (d, J = 2.5 Hz, 1, pyridine H-6).

1-(4-Fluorophenyl)-4-[4-(5-hydroxy-2-pyridinyl)-1-piperazinyl]-1-butanol (10) from 16—The procedure described for the preparation of **10** from **17** was repeated with 60 mg (0.17 mmol, 1 equiv) of **16** and 26 mg (0.7 mmol, 8 equiv) of lithium aluminum hydride to afford, after chromatography on silica gel using methanol:chloroform (1:5, v/v), 38 mg (63%) of **10**.

Results and Discussion

We have identified two pyridine ring hydroxylated metabolites of azaperone in basic extracts of horse urine treated with β-glucuronidase to hydrolyze glucuronic acid conjugates. The metabolites were identified by chemical synthesis, GC/MS analysis, ^{1}H NMR analysis, and TLC. These metabolites are identified as 1-(fluorophenyl)-4-[4-(5-hydroxy-2-pyridinyl)-1-piperazinyl-]-1-butanol, designated as 5′-hydroxyazaperol (**10**), and 1-(fluorophenyl)-4-[4-(5-hydroxy-2-pyridinyl)-1-piperazinyl]-1-butanone, designated as 5′-hydroxyazaperone (**16**).

Isolation and Characterization of Azaperone Metabolites from Urine—Azaperone metabolites, 5′-hydroxyazaperol (**10**) and 5′-hydroxyazaperone (**16**), were isolated from urine samples subjected to hydrolysis with β-glucuronidase/sulfatase from limpets (*Patella vulgata*) and extracted under alkaline conditions. Both metabolites exhibited intense blue fluorescence when illuminated under 254- or 350-nm UV light and both metabolites reacted with Folin-Denis and Dragendorff spray reagents, suggesting the presence of an aromatic hydroxyl group and a basic nitrogen atom. Both metabolites were detected by TLC analysis of extracts of the 0−1 h through the 7−8 h urine samples; the intensity of the metabolite spots decreased in extracts of urine samples collected through 8 h, and only 5′-hydroxyazaperol was detectable in a 5-mL extract of the 24-hour sample. Neither azaperone nor azaperol was detected by TLC of any extract (the limits of detection for these analytes in alkaline extracts of urine were approximately 100 ng/mL). Azaperone metabolites **10** and **16** were not detected in the extracts of alkaline urine unless the urine was first treated with β-glucuronidase. No evidence for other azaperone metabolites was detected in any extract.

Mass spectral analysis (Figure 2) of the trimethylsilyl (TMS) derivative (**19**) of the more polar metabolite under electron-impact ionization conditions indicated an apparent molecular ion of m/z 489 and a base peak ion of m/z 195, both of which provided valuable diagnostic evidence for the proposed structure of 5′-hydroxyazaperol (**10**). First, the increased molecular mass (162 amu) of the base peak ion of the metabolite (**16**) relative to that of azaperone was consistent with the addition of an oxygen atom, reduction of the carbonyl group, and addition of two TMS groups as in the derivative (**19**). Secondly, in an analysis of the fragmentation pattern of azaperone, Rauws *et al.* proposed that the base peak ion at m/z 107 represented an ion from 2-(methylamino)pyridine.[4] Consequently, the base peak ion at m/z 195 displayed by the metabolite indicated that the pyridyl ring in the metabolite had undergone the addition of oxygen and TMS formation. Third, an analysis of the high-resolution mass spectrum of the TMS derivative (**18**) of azaperol indicated that the ion at m/z 197.0801 (calculated 197.0798) represented a $C_{10}H_{14}OFSi$ fragment from the butyrophenone portion of azaperol. This ion was also present in the more polar metabolite and was therefore consistent with the proposed reduction of the carbonyl group of azaperone in the metabolite. In summary, the mass spectral analysis of the TMS derivative (**19**) of the more polar metabolite was consistent with hydroxylation of the pyridine ring and reduction of the carbonyl group of azaperone.

The position of pyridine ring hydroxylation was hypothetically assigned to C-5 on the basis of earlier studies of pyridine ring hydroxylation of pyrilamine and tripelennamine in horses.[10] This assignment was ultimately confirmed in a comparison of the ^{1}H NMR spectra of the isolated more polar metabolite with the authentic standard of 5′-hydroxyazaperol (**10**) produced by an unambiguous synthesis described below. Comparison of thin layer chromatographic data from seven solvent systems and GC/MS data for the isolated metabolite and the authentic standard of 5′-hydroxyazaperol corroborated the structural assignment.

The mass spectrum (Figure 3) of the TMS derivative of the second, less polar metabolite exhibited an apparent molecular ion of m/z 415, no significant ion at m/z 197, and a base peak ion at m/z 195. The net increase of 88 amu in the parent ion relative to that of azaperone again suggested the addition of oxygen and one TMS group. The ion at m/z 195 indicated that oxidation occurred on the pyridine ring just as in metabolite **10**, and the lack of an ion at m/z 197 indicated that the carbonyl group was not reduced. On this basis, the tentative structure of the second metabolite was assigned as 5′-hydroxyazaperone (**16**). In support of this assignment, the parent ion at m/z 415 was consistent with a TMS derivative in which 5′-hydroxyazaperone (**16**) underwent silylation on the hydroxyl group of the pyridine ring. Also consistent with the presence of a carbonyl group in the second metabolite was the absence of the m/z 197 ion characteristic of the azaperol

structures. The evidence in support of the tentative structural assignment of 5′-hydroxyazaperone (**16**) as the less polar metabolite was subsequently confirmed in a comparison of the mass spectra (Figure 3) of the TMS ether derivatives of the isolated metabolite and authentic standard of **16**.

The position of pyridine ring hydroxylation was tentatively assigned to C-5 on the basis of our assignment for 5′-hydroxyazaperol. This assignment was ultimately confirmed in a comparison of the ^{1}H NMR spectra of the isolated less polar metabolite with the authentic standard of 5′-hydroxyazaperone (**16**) produced by an unambiguous synthesis described below. The ^{1}H NMR analysis also confirmed the presence of the carbonyl group in this metabolite.

Comparison of thin layer chromatographic data from seven solvent systems and GC/MS data for the isolated metabolite and the authentic standard of 5′-hydroxyazaperol corroborated the structural assignment.

Azaperone metabolites **10** and **16** were detected by TLC in extracts of glucuronidase-treated urine, indicating that the metabolites were excreted as glucuronic acid conjugates (the preparation of β-glucuronidase from *P. vulgata* contains sulfatase activity that is inhibited by 0.1 M phosphate buffer).[9] These metabolites were not detected by TLC analysis when the activity of β-glucuronidase was competitively inhibited by the addition of D-saccharic acid 1,4-lactone. Furthermore, the yields of 5′-hydroxyazaperol (**10**) and 5′-hydroxyazaperone (**16**) based on semiquantitative TLC analysis were not diminished by inhibition of sulfatase activity by substitution of phosphate buffer for acetate buffer during β-glucuronidase/sulfatase treatment.[9] These findings indicated that both metabolites were eliminated in urine as glucuronic acid conjugates, in agreement with the previous observations reported by Chui *et al*.[7] The identities of the metabolic products of azaperone in horse urine determined by Chui *et al*.[7] and reported in this study are summarized in Figure 1.

Chemical Synthesis of Azaperone Metabolites—Tentative structural assignments based on mass spectral and NMR data collected on the two metabolites isolated from urine were confirmed by the unambiguous synthesis of each metabolite.

In an approach to the metabolite that paralleled the synthesis of azaperone,[11] we examined a route that involved a nitro-substituted azaperone as the key intermediate (**13**). In this case, the nitro substituent would serve as a progenitor of the 5-hydroxy group. The reaction of piperazine (**11**) with 2-bromo-5-nitropyridine afforded 1-(5-nitro-2-pyridinyl)piperazine (**13**). Efforts, however, to couple this derivative with 1-(4-fluorophenyl)-4-chloro-1-butanone according to the published procedure furnished 1-(fluorophenyl)-4-[4-(5-nitro-2-pyridinyl)-1-piperazinyl]-1-butanone (**13**) in very poor yield.[9] However, the reverse sequence involving the coupling of piperazine (**11**) to 1-(4-fluorophenyl)-4-chloro-1-butanone furnished 1-fluorophenyl-4-(1-piperazinyl)-1-butanone (**3**), and the subsequent coupling with 5-nitro-2-bromopyridine furnished the desired product (**13**) in good overall yield. The reduction of the nitro group in **13** to the amine (**14**), diazotization of **14**, and hydrolysis of the diazonium salt (**15**) gave the metabolite 5′-hydroxyazaperone (**16**), in an overall yield of 23% for this three-step process.

Preparation of the acetate (**17**) provided a derivative of the metabolite that was readily characterized, and the reduction of either the metabolite (**16**) or its acetate (**17**) furnished the other metabolite, 5′-hydroxyazaperol (**10**).

Detection and Confirmation of Azaperone Administration to Horses—Detection of azaperone administration to horses was accomplished by TLC analysis of basic extracts of 5-mL aliquots of β-glucuronidase/sulfatase-treated urine samples collected from 0 to 1 through 24 h after intravenous doses of 40 mg per horse (0.071−0.084 mg/kg of body weight). Since the dose used in this investigation was considerably less than doses used to produce tranquilization (0.40 to 0.80 mg/kg im)[3] or those reported to produce excitement and ataxia (0.29 to 0.57 mg/kg iv) in horses,[4] this method is suitable for detecting the administration of azaperone at doses that might affect the performance of the horse.

Confirmation of azaperone administration to horses was accomplished by GC/MS identification of 5′-hydroxyazaperol (**10**) or 5′-hydroxyazaperone (**16**). The presence of 5′-hydroxyazaperol, the more abundant metabolite on the basis of semiquantitative TLC analysis, was confirmed as its TMS ether derivative (**19**) by electron-impact ionization GC/MS of the basic extract of β-glucuronidase/sulfatase-treated urine samples collected through 24 h after intravenous drug administration.

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