Identification of Metabolites of Azaperone in Horse Urine

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ABSTRACT

Two metabolites of the tranquilizer, azaperone (Stresnil™),in horse urine were identified by a combination of independent chemical synthesis and isolation in combination with gas chromatography-mass spectrometry and nuclear magnetic resonance spectrometry to be 1-(fluorophenyl)-4-[4-(5-hydroxy-2-pyridinyl) -1-piperazinyl]-1-butanol (5'-hydroxyazaperol) and 1-(fluorophenyl) -4-[4-(5-hydroxy-2-pyridinyl)-1-piperazinyl]-1-butanone (5'-hydroxyazaperone). Thin layer chromatographic and enzyme-linked immunoassay tests were developed to detect these metabolites in horse urine.

INTRODUCTION

The use of drugs to affect performance of animals and humans participating in athletic competition is a continuing problem throughout the entire scope of sporting events. Many sports regulatory bodies such as the National Collegiate Athletic Association, the American Horse Shows Association, and the Association of Racing Commissioners International, Inc. have established rules prohibiting the presence of certain drugs or their metabolites in test samples collected from athletic competitors in an effort to prevent unfair, dangerous, or inhumane use of drugs. Therefore, methods permitting the detection and unequivocal identification of drugs and drug metabolites in test samples such as blood and urine are needed to enforce these rules.

Racing commissions and horse shows associations generally prohibit the presence of narcotics, stimulants, depressants, tranquilizers, and local anesthetics as well as their metabolites in test samples. Azaperone (Stresnil™, Janssen Pharmaceutica, Beerse, Belgium; 1-(4-fluorophenyl)-4-[4-(2-pyridinyl)-1-piperazinyl]-1-butanone; compound (1) in Figure 1) is a butyrophenone tranquilizer approved for use in swine to prevent stress-related injuries, loss of weight, and mortality during transport. However, there have been recent reports of azaperone use in performance horses (CanTest, B.C., Canada and Dalare Laboratories, Philadelphia, private correspondence). The rationale for administration of azaperone to horses is not entirely clear since it produces tranquilization in horses after intramuscular doses of 0.40-0.80 mg/kg of body weight (Serrano and Lees, 1976) and excitement and ataxia after intravenous doses of 0.29-0.57 mg/kg of body weight (Dodman and Waterman, 1979).

Reduction of the carbonyl group in azaperone (1) to azaperol (2) in Figure 1 is a major metabolic route for azaperone in swine (Rauws *et al.*, 1976). 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

(Heykants *et al.*, 1971). However, analysis of urine samples collected from experimental horses administered azaperone at various laboratories has indicated azaperone (1) is not present at significant concentrations and other known azaperone metabolites, including azaperol (2), are not the major metabolites in horse urine. Therefore, the purpose of this study was to identify azaperone metabolites in horse urine and develop effective thin layer chromatographic and enzyme-linked immunoassay tests so that the administration of azaperone to competition horses could be detected.

MATERIALS AND METHODS

Chemicals

Azaperone hydrochloride injectable solution was purchased from Pitman-Moore, Inc., Washington Crossing, New Jersey, and other chemicals including D-saccharic acid 1,4-lactone monohydrate and deuterated methylene chloride (99.6% isotopic purity) were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Type L-II β-glucuronidase containing sulfatase from *Patella vulgata* was purchased from Sigma Chemical Co., St. Louis, Missouri. Preparative TLC plates (silica gel 60 F_{254}, 20 x 20 cm, 0.5 mm thickness) were purchased from Analtech, Newark, Delaware. Analytical TLC plates (silica gel 60 F_{254}, 5 x 10 cm, 0.25 mm thickness) were purchased from EM Science, Inc., Cherry Hill, New Jersey. Combustion analyses were performed by Atlantic Microlabs, Norcross, Georgia.

Synthesis of Reference Compounds

1-(4-Fluorophenyl)-4-[4-(2-pyridinyl)-1-piperazinyl]-1-butanol ("Azaperol") (2). To a solution of 1.31 g (4 mmol, 1 eq) 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 eq) of lithium aluminum hydride in 10 mL of ether. The mixture was stirred at 25°C for 30 minutes and carefully hydrolyzed by adding 0.5 mL of water dropwise with stirring over a 10 minute 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}; ^1H NMR ($CDCl_3$) k 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 ($CDCl_3$) k 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 calcd for $C_{19}H_{24}FN_{30}$ 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 eq) of (2) in

5 mL of anhydrous pyridine was added 698 mg (645 μL, 6.84 mmol, 2 eq) of acetic anhydride followed by 50 mg of 4-(N,N-dimethylamino) pyridine (DMAP) at 0°C. The mixture was stirred at 0°C for 2 hours and at 25°C for 18 hours. The solvent was removed under reduced pressure, and the residue was dissolved in water. The solution was diluted with a saturated sodium bicarbonate solution 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}; ^1H NMR ($CDCl_3$) k 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 ($CDCl_3$) k 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. Calcd. 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 eq) of (2) in 10 mL of acetic acid at 25°C was slowly added a solution of 163 mg (1.02 mmol, 1 eq) of bromine in 2 mL of acetic acid. The mixture was stirred for 30 minutes. 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}; ^1H NMR ($CDCl_3$) k 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 ($CDCl_3$) k 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 eq) of (5) in 25 mL of acetic acid at 25°C was slowly added a solution of 517 mg (3.23 mmol, 1 eq) of bromine in 25 mL of acetic acid. The mixture was stirred for 15 minutes and concentrated under reduced pressure. The residue was diluted with water, made basic by addition of a saturated sodium bicarbonate solution, 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 ($CDCl_3$) k 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 ($CDCl_3$) k 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 calcd for $C_{21}H_{25}BrFN_3O_2$ 449.1116, found 449.1115.

1-(4-Fluorophenyl)-4-[4-(5-bromo-2-pyridinyl)-1-piperazinyl]-(tert-butyldimethylsilyloxy)butane (8). To a solution of 408 mg (1 mmol, 1 eq) of (6) in 2 mL of anhydrous N,N-dimethyl-formamide was added 170 mg (2.5 mmol, 2.5 eq) of imidazole. The mixture was stirred at 25°C for 5 minutes, and 226 mg (1.5 mmol, 1.5 eq) of tert-butyldimethylsilyl chloride was added. The solution was stirred at 25°C for 18 hours. 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 1:1 EtOAc-hexane to afford 450 mg (86%) of (8): IR (TF) 2952, 2885, 2856, 1605, 1584, 1548, 1509 cm^{-1}; ^1H NMR ($CDCl_3$) k -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 ($CDCl_3$) k -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. Calcd. 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 eq) of piperazine (11) and 1.02 g (5 mmol, 1 eq) of 2-bromo-5-nitropyridine in 5 mL of isoamyl alcohol was added 530 mg (5 mmol, 1 eq) of anhydrous sodium carbonate. The mixture was refluxed under a Dean Stark trap for 2 hours. The mixture was cooled; the solids were removed by filtration; and the clear filtrate was concentrated and chromatographed on silica gel using 1:5 methanol-chloroform to afford 880 mg (85%) of (12): IR (KBr) 3346, 2953, 2900, 2834, 1607, 1568, 1525 cm^{-1}; ^1H NMR ($CDCl_3$) k 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 eq)

of (12) and 8.02 mg (4 mmol, 2 eq) of 4-chloro-1-(4-fluorophenyl) -1-butanone in 10 mL of n-butanol was added 212 mg (2 mmol, 1 eq) of anhydrous sodium carbonate and 150 mg (1 mmol, 0.5 eq) of sodium iodide. The mixture was refluxed under a nitrogen atmosphere for 20 hours, 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 1:19 methanol-chloroform to afford 180 mg (24%) of (13): IR (KBr) 2949, 1689, 1595, 1510 cm^{-1}; ^1H NMR ($CDCl_3$) k 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 ($CDCl_3$) k 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. Calcd. 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 eq) of 4-chloro-1-(4-fluorophenyl) -1-butanone and 1.72 g (20 mmol, 2 eq) of piperazine (11) in 10 mL of isoamyl alcohol was added 1.06 g (10 mmol, 1 eq) of anhydrous sodium carbonate. The mixture was refluxed under a Dean Stark trap for 3 hours. 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 1:8:40 ammonium hydroxide-methanol-chloroform to afford 1.78 g (71%) of (3): IR (KBr) 3435, 2954, 2823, 1677, 1600, 1507 cm^{-1}; ^1H NMR ($CDCl_3$) k 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 eq) of (3) and 715 mg (3.52 mmol, 1 eq) of 2-bromo-5-nitro-pyridine in 5 mL of isoamyl alcohol was added 373 mg (3.52 mmol, 1 eq) of anhydrous sodium carbonate. The mixture was refluxed, with continuous removal of water under a Dean Stark trap, for 3 hours. 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 1:19 methanol-chloroform 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 eq) 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 hours. 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 H_2SO_4. The mixture was cooled to 0°C; 83 mg (1.2 mmol, 1.2 eq) of sodium nitrite in 1 mL of water was added dropwise; and stirring was continued at 0-5°C for 15 minutes. 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 hours. 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 1:5 methanol-chloroform to afford 80 mg (23%) of (16): IR (KBr) 3540 (br), 2835, 1680, 1590 cm^{-1}; ^1H NMR ($CDCl_3$) k 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 ($CDCl_3$) k 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 (76 fm, 1 eq) of (16) in 0.5 mL of pyridine was added 15.5 mg (15 µL, 152 µm, 2 eq) of acetic anhydride and 2 mg (15 fm, 0.2 eq) of 4-(N,N-dimethylamino)pyridine. The mixture was stirred at 25°C for 18 hours. The solvent was removed under reduced pressure. The residue was dissolved in chloroform, washed with saturated sodium bicarbonate solution and brine, dried over anhydrous sodium bicarbonate, concentrated, and chromatographed on silica gel using 1:10 methanol-chloroform to afford 22 mg (76%) of (17): IR (KBr) 1750, 1680, 1595 cm^{-1}; ^1H NMR ($CDCl_3$) k 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. Calcd. 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 eq) of (17) in 3 mL of 2:1 ether-tetrahydrofuran at 0°C was slowly added a solution of 8 mg (0.20 mmol, 8 eq) of lithium aluminum hydride in 1 mL of ether. The mixture was stirred at 25°C for 30 minutes 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 chromato-

graphed on silica gel using 1:5 methanol-chloroform to afford 17 mg (94%) of (10): IR (KBr) 3432, 3104, 2937, 1605, 1577, 1498 cm^{-1}; ^1H NMR ($CDCl_3$) k 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), 6.92-7.10 (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 eq) of (16) and 26 mg (0.7 mmol, 8 eq) of lithium aluminum hydride to afford, after chromatography on silica gel using 1:5 methanol-chloroform, 38 mg (63%) of (10).

Administration of Azaperone to Test Horses

Five mature female horses 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 two hours before and eight hours 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), was administered intravenously to each horse at a total dose of 40 mg/horse (0.071 to 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 hours after drug administration. Urine samples were 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 thin layer chromatography.

Extraction of Alkalinized Urine. A 9-mL urine aliquot from each collection interval was alkalinized with 2 mL of a sodium carbonate (1 M)/bromothymol blue (200 mg/L) solution in a disposable screw cap culture tube. The urine was then extracted with 5 mL of methylene chloride-isopropanol (75:25, by vol.) by end over end rotation at 20 rpm for 5 minutes. The phases were separated by centrifugation at 1000 *g* for 5 minutes. The aqueous phase was removed by a vacuum aspirator, and the organic phase was transferred to a clean 5-mL conical centrifuge tube. The extracts were then evaporated under a flow of nitrogen in a water bath at 40

to 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 samples were then extracted with 5 mL of methylene chloride-isopropanol (75:25, by vol.) by end over end rotation at 20 rpm for 5 minutes. The phases were separated and the organic phases concentrated as described above.

Extraction of Acidic 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 HCl). The samples were then extracted with 5 mL of methylene chloride-petroleum ether (10:1, by vol.) by end over end rotation at 20 rpm for 5 minutes. 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 M acetate buffer (pH 5.0) and 1 mL of β-glucuronidase/sulfatase solution (5,000 units of β-glucuronidase/mL in distilled water) was added. Each tube was vortex mixed and then placed in an incubator at 65°C for 4 hours.

After cooling, 0.5 mL of 10% ascorbic acid in water was added to each tube, and the pH was adjusted to 8.5-9.2 with 6 N HCl or a solution of concentrated ammonia (sp. gr. 0.88) and distilled water (1:1, by vol.) as needed. The samples were extracted with 5.0 mL of methylene chloride-isopropanol (10:1, by vol.) by end over end rotation at 20 rpm for 5 minutes and centrifuged at 1000 *g* for 5 minutes. 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 H_2SO_4, the organic phases were discarded, and the aqueous phases were transferred to clean tubes.

Another 0.2 mL aliquot of 10% ascorbic acid was added to each tube. The pH was adjusted, with concentrated ammonia to 8.5-9.2 and 5 mL of methylene chloride-isopropanol (10:1, by vol.) was added to each tube. The samples were mixed, centrifuged, and the organic layers were transferred to clean tubes and concentrated under nitrogen as described above.

Verification of Glucuronic Acid Conjugation by Glucuronidase and Sulfatase Inhibition

The presence of glucuronic acid conjugates of azaperone metabolites was verified by the inhibiting glucuronidase by addition of D-saccharic acid 1,4-lactone monohydrate and inhibiting sulfatase by substitution of phosphate buffer for acetate buffer.

Three 5 mL-aliquots of the 0 to 1 hour 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 (5000 units of β-glucuronidase/mL). Then 0.1 mL of water was added to tube #1, 0.1 mL of 8.1 mM D-saccharic acid 1,4-lactone monohydrate (final concentration = 1.0 mM) to tube #2, and 0.1 mL of 81 mM D-saccharic acid 1,4-lactone monohydrate (final concentration = 10 mM) to tube #3. The mixtures were incubated at 40°C for 3 hours and extracted as described above.

Sulfatase was inhibited by substituting phosphate buffer for acetate buffer. Aliquots (5 mL) of the 0 to 1 hour urine samples were acidified by the addition of 2 mL of 0.4 M KH_2PO_4 buffer (pH 5.0) and mixed with 1 mL of β-glucuronidase/sulfatase (5000 units of β-glucuronidase/mL). The final solution containing 0.1 M phosphate was incubated at 40°C for 3 hours and then extracted as described above.

Analytical Thin Layer Chromatography

Residues obtained from the various urine extracts were subjected to analytical TLC on Merck precoated silica gel 60 F-254 plates (0.25 mm thickness). Each residue was dissolved in 20 μL of ethyl acetate and spotted directly on the origin line of 7 different TLC plates using a Hamilton 10-μL syringe. Each thin layer chromatography plate was developed a 5-cm distance in one of each of the following solvent systems: (I) methanol-ammonia solution (sp. gr. 0.88) (100:1.5, by vol.); (II) ethyl acetate-methanol-ammonia solution (sp. gr. 0.88) (80:15:5, by vol.); (III) chloroform-ethanol (9:1, by vol.); (IV) chloroform-ethanol (19:1, by vol.); (V) chloroform-cyclohexane-acetic acid (4:4:2, by vol.); (VI) chloroform-methanol-propionic acid (72:18:10, by vol.); (VII) ethyl acetate-methanol-acetic acid (8:1:1, by vol.).

The locations of the azaperone metabolites were visualized by fluorescence under 350-nm UV light, absorption under 254-nm UV light, and color formation after sequential application of Folin/Dennis and Dragendorff spray reagents (Stahl 1967).

Preparative Isolation of Metabolites for Structural Analyses.

Preparative isolation of azaperone metabolites extracted from the 0 to 1 hour urine samples was performed using the β-glucuronidase hydrolysis method described above. The hydrolysis was carried out on 200 mL of urine in forty 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 x 20 cm silica gel GF preparative thin layer chromatography plates (0.5 mm thickness,

Analtech, Newark, Delaware). The extract in ethyl acetate was streaked along the origin, and the TLC plate was developed 10 cm in solvent system VI. Metabolite zones were visualized under 350-nm and 254-nm UV light and then collected with a Kontes 3-mL zone collector. 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 dissolved in 150 µL of ethyl acetate-methanol (2:1, by vol.) and rechromatographed in solvent system VI. The metabolite zones were visualized and collected as before. The TLC isolation procedure was repeated using solvent system III and then using solvent system II. 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 methylene chloride by vortex mixing for 30 seconds and then centrifuging to separate the layers. The methylene chloride solution was transferred to another clean conical centrifuge tube, and the aqueous phase was extracted again with another 2.5 mL of methylene chloride. The methylene chloride extracts were combined and then concentrated under a flow of nitrogen. The isolated metabolites were then dried in a vacuum desiccator over phosphorous pentoxide.

All isolation and purification was done using TLC plates that were thoroughly cleaned before use by developing them twice in HPLC-grade methanol and twice in HPLC-grade acetonitrile.

Derivatization of Metabolites for Gas Chromatography-Mass Spectrometry.

Metabolites were derivatized with 20 µL of N,O-bistrimethylsilylacetamide (BSTFA; Pierce, Rockford, Illinois) in a capped, conical tube for 30 minutes at 65°C. The mixture was diluted with 20 µL of ethyl acetate before GC-MS analysis.

Low Resolution Gas Chromatography-Mass Spectrometry.

The gas chromatograph-mass spectrometer 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 sample extracts were injected via the splitless injector at 280°C on to a 15 m x 0.251 mm i.d. DB-1 capillary column (0.25 µm film thickness, J & W Scientific, Rancho Cordova, California) at an initial temperature of 150°C. After 1 minute, the temperature of the column oven was increased at a rate of 20°C/minute to a final temperature of 280°C and was maintained at that temperature for 12.5 minutes. Helium was used as the carrier gas at a flow rate of 1 mL/minute. The mass spectrometer was operated under electron-impact ionization conditions at a source potential of 70 eV. Perfluorotributylamine was used as the reference compound.

High Resolution Gas Chromatography-Mass Spectrometry.

High resolution GC-MS analysis was done at the Ohio State University Physical Chemistry Instrument Center. The gas chromatograph was a Hewlett-Packard model 5890 fitted with a 30 m x 0.251 mm i.d. DB-1 capillary column (0.25 μm film thickness, J & W Scientific, Rancho Cordova, California). The injector was at 280°C, and helium was the carrier gas (1 mL/min). The column oven temperature started at 100°C, was maintained for 3 minutes and was then increased at a rate of 15°C/min to 280°C. The mass spectrometer was a VG model 70-250S double focusing magnetic sector instrument. All spectra were obtained under electron-impact ionization conditions at a source potential of 70 eV at 200°C. Perfluorokerosene was used as the reference compound.

Nuclear Magnetic Resonance Spectrometry.

The purified metabolite residues were dissolved in 1-2 mL of HPLC grade ethyl acetate and transferred to 5 x 250 mm NMR tubes (Wilmad, Buena, New Jersey). The ethyl acetate was concentrated to dryness with a flow of nitrogen at room temperature. The tubes were then placed in a vacuum desiccator (phosphorus pentoxide as desiccant) for two days. The residues in the NMR tubes were dissolved in CD_2Cl_2 for ^1H NMR analysis with a Bruker AM-500 system.

ELISA Test Method

The ELISA tests developed here are similar to the ELISA formats used in a published report by Stanley et al., 1991[10]. The anti-azaperone antibody was coated to flat bottom wells (Costar©, Cambridge, Massachusetts) as described by Voller, 1976[11]. Azaperone was linked to horse radish peroxidase (HRP) to give rise to a covalently linked drug-HRP complex by the method described by Rowley et al., 1975[7]. All assays were performed at room temperature. The assay was started by adding 20 μL of the standard, test, or control samples to each well, along with 180 μl of the drug-HRP solution. During the test, the presence of drug in the sample competitively prevented the binding of drug-HRP complex to the antibody. Since the HRP enzyme was responsible for the color-producing reaction in the ELISA, the concentration of drug in the sample was inversely related to the optical density of the test well. The optical density (OD_{650}) of the test wells was read at a wavelength of 650 nm with an automated microplate reader (Bio-Tek Instruments, Winooski, Vermont) approximately 60 minutes after addition of substrate.

RESULTS AND DISCUSSION

We have identified two azaperone metabolites in horse urine by chemical synthesis, gas chromatography-mass spectrometry, ^1H

nuclear magnetic resonance spectrometry, and thin-layer chromatography. These metabolites are identified as 1-(fluorophenyl)-4-[4-(5-hydroxy-2-pyridinyl)-1-piperazinyl-]-1-butanol, designated as 5'-hydroxyazaperol (10) in Figure 3, and 1-(fluorophenyl)-4-[4-(5-hydroxy-2-pyridinyl)-1-piperazinyl]-1-butanone, designated 5'-hydroxy-azaperone (16) in Figure 3.

Isolation and Characterization of Azaperone Metabolites from Urine. Azaperone metabolites, 5'-hydroxyazaperol (10) and 5'-hydroxy-azaperone (16), were isolated from urine samples subjected to hydrolysis with β-glucuronidase from *Patella vulgata* and extracted under alkaline conditions. Both metabolites exhibited intense fluorescence when illuminated under 254-nm 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 of the extract of the 0 to 1 hour urine sample, the intensity of the metabolite spots decreased through 8 hours, and only 5'-hydroxyazaperol was detectable in a 5-mL extract of the 24-hour sample. Neither azaperone (1) nor azaperol (2) was detected by TLC of any extract (limits of detection were approximately 100 ng/mL). Azaperone metabolites (10) and (16) were not detected in the extracts from alkaline urine (solvent systems I and II), neutral urine (solvent systems I, II, and VI), or acidic urine (solvent systems II, III, and VI).

Mass spectral analysis of the trimethylsilyl (TMS) derivative (19) in Figure 4 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 of the parent ion (162 amu) of the metabolite (16) relative to azaperone (1) 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 (1), Rauws et al. (1976) proposed that the base peak at m/z 107 ion represented an ion from 2-(methylamino)pyridine. Consequently, the base peak 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 as shown in Figure 4. Third, an analysis of the mass spectrum of the trimethylsilyl derivative (18) of azaperol indicated that the ion at m/z 197.0801 (calcd. 197.0798) represented a $C_{10}H_{14}OFSi$ fragment (Figure 4) 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 based on earlier studies of pyridine ring hydroxylation of pyrilamine and tripelennamine in horses (unpublished data). This assignment was ultimately confirmed in a comparison of the ^1H NMR spectra of the isolated more polar metabolite with the authentic sample of 5'-hydroxyazaperol (10) produced by an unambiguous synthesis described below. Further comparisons of the isolated and authentic samples of 5'-hydroxyazaperol (10) were extended to analytical TLC in the seven solvent systems (Table 1) and to GC-MS comparisons (Figure 1). These data corroborated this structural assignment.

The mass spectrum 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 azaperone (1) 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 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'-hydroxy-azaperone (16) as the less polar metabolite was subsequently confirmed in a comparison of the mass spectra (Figure 4) of the isolated and authentic samples of (16).

The position of pyridine ring hydroxylation was tentatively assigned to C-5 based on our assignment for 5'-hydroxyazaperol. This assignment was ultimately confirmed in a comparison of the ^1H NMR spectra of the isolated less polar metabolite with the authentic sample of 5'-hydroxyazaperone (16) produced by an unambiguous synthesis described below. The ^1H NMR analysis also confirmed the presence of the carbonyl group in this metabolite. Further comparisons of the isolated and authentic samples of 5'-hydroxyazaperone (16) were extended to analytical TLC in the seven solvent systems (Table 1) and to GC-MS comparisons (Figure 4). These data corroborated this structural assignment.

Azaperone metabolites were detected by TLC in extracts of glucuronidase/sulfatase treated urine indicating that the metabolites were excreted as glucuronic acid or sulfate conjugates. No metabolites were detected when the activity of β-glucuronidase was inhibited by the addition of D-saccharic acid 1,4-lactone. Furthermore, the yields of 5'-hydroxyazaperol (10) and 5'-hydroxyazaperone (16) were not affected by inhibition of sulfatase by the substitution of 0.1 M phosphate buffer for the acetate buffer. These findings indicate that both metabolites were eliminated in urine as glucuronic acid conjugates.

Chemical Synthesis. In order to confirm tentative structural assignments based on mass spectral and NMR data collected on the metabolites isolated from urine, we undertook the unambiguous synthesis of each metabolite. Initial efforts to prepare the metabolite, 5'-hydroxyazaperol (10), from azaperone (1) itself involved a straightforward sequence of reactions shown in Figure 2 in which the key step was the conversion of a 3-bromopyridine derivative to the desired 3-hydroxypyridine subunit of the metabolite (10). Although the yield in this crucial oxidation step was expected to be low, this would not be a significant problem since we required only a small sample for confirmation purposes and since the overall number of steps from azaperone (1) to the metabolite (10) was small. The reduction of the carbonyl group in azaperone (1) and the regioselective bromination of the alcohol (2) or the acetate (5) provided the pyridyl bromides (6) and (7), respectively. We anticipated that exposure of the acetate (7) to an excess of n-butyllithium would remove the acetate protecting group and metalate the 3-bromopyridine group to give an intermediate 3-lithiopyridine (9). Exposure of this intermediate to oxygen and reduction of the intermediate hydroperoxide would secure the metabolite (10). However, all efforts to effect this transformation using either (7) or (6) proved fruitless. The principal product was the reduced product, azaperol (2) rather than the desired metabolite, 5'-hydroxyazaperol (10).

In an alternate approach to the metabolite that paralleled the synthesis of azaperone (1), we examined the route in Figure 3 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 (12). 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. 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).

ELISA test performance. A standard curve for the azaperone ELISA test indicates that addition of 400 pg of azaperone per mL of buffer to the system produced about 50 percent inhibition (Figure

5). Increasing concentrations of azaperone increased the inhibition in a sigmoidal manner, with half-maximal inhibition occurring at approximately 400 pg of azaperone per mL. The antibody showed little cross-reactivity with haloperidol or droperidol. Cross-reactivities for pimozide and spiperone were also evaluated (Figure 6), and the antibody did not have significant affinity for these drugs. additionally, cross-reactivity studies were performed on the two metabolites which were described earlier. The metabolite 5'-hydroxyazaperol showed no significant affinity for the antibody, while the 5'-hydroxyazaperone metabolite displayed similar cross-reactivity to that of the parent compound azaperone with a I-50 of 0.8 ng/mL.

The ability of this ELISA test to detect azaperone in the urine of horses dosed with the drug was also evaluated. Azaperone was administered intravenously at 40 mg/horse to each of five horses. Azaperone equivalents were readily detectable in the urine of these horses (Figure 7). At the 40 mg/horse dose, inhibition of the ELISA reaction in urine was essentially complete from 1 hour through 8 hours post-dose. By 24 hours after dosing, however, the reaction was only about 80% inhibited and at 48 hours inhibition was no longer detectable.

Detection and Confirmation of Azaperone Administration to Race Horses. Detection of the azaperone administration to horses was accomplished by TLC and ELISA testing of urine samples collected for 24 hours after intravenous doses of 40 mg per horse or 0.071 to 0.084 mg/kg of body weight. Since this dose is less than doses used to produce tranquilization in horses (0.40 to 0.80 mg/kg intramuscularly) or those reported to produce excitement and ataxia (0.29 to 0.57 mg/kg intravenously), either of these methods would appear suitable for detecting the administration of azaperone doses that might affect performance of the horse. Both the TLC and the ELISA method had adequate sensitivity to detect azaperone metabolites in urine samples collected for 24 hours after administration of the 40 mg dose. Furthermore, both methods are suitable for batch analysis thereby reducing the time and personnel required for mass screenings of large numbers of samples.

Confirmation of azaperone administration to horses was accomplished by GC-MS identification of 5'-hydroxyazaperol or 5'-hydroxyazaperone. Confirmation of 5'-hydroxyazaperol, the more abundant metabolite, by electron-impact ionization GC-MS of the basic extract of urine samples collected through 24 hours after drug administration was also possible. Therefore, the presence of azaperone metabolites detected by either TLC or ELISA was confirmed by GC-MS.

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Table 1.

Thin-Layer Chromatographic R_f Values for Azaperone (1), Azaperol (2), 5'-Hydroxyazaperol (10), and 5'-Hydroxyazaperone (16).

	Solvent Systems						
	I	II	III	IV	V	VI	VII
Azaperone (1)	0.88	0.90	0.58	0.24	0.00	0.68	0.08
Azaperol (2)	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

Solvent systems: (I) methanol-ammonia solution (sp. gr. 0.88) (100:1.5, by vol.); (II) ethyl acetate-methanol-ammonia solution (sp. gr. 0.88) (80:15:5); (III) chloroform-ethanol (9:1, by vol.); (IV) chloroform-ethanol (19:1, by vol.); (V) chloroform-cyclohexane-acetic acid (4:4:2, by vol.); (VI) chloroform-methanol-propionic acid (72:18:10, by vol.); (VII) ethyl acetate-methanol-acetic acid (8:1:1, by vol.).

Figure 1.

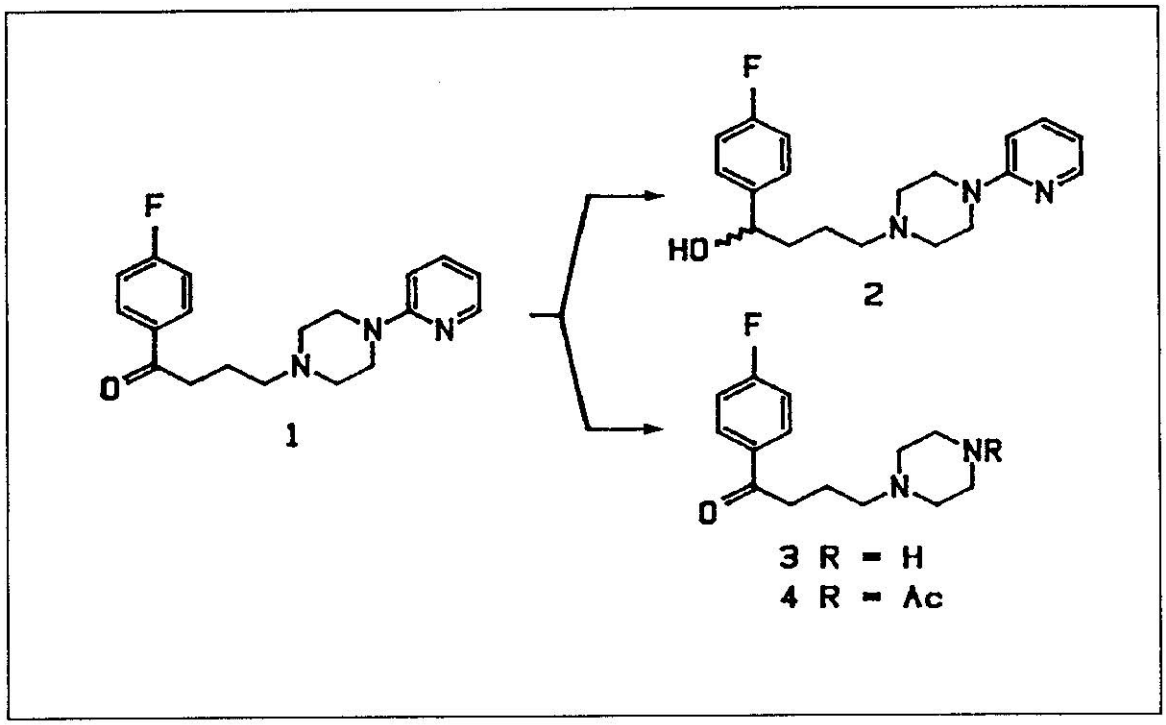
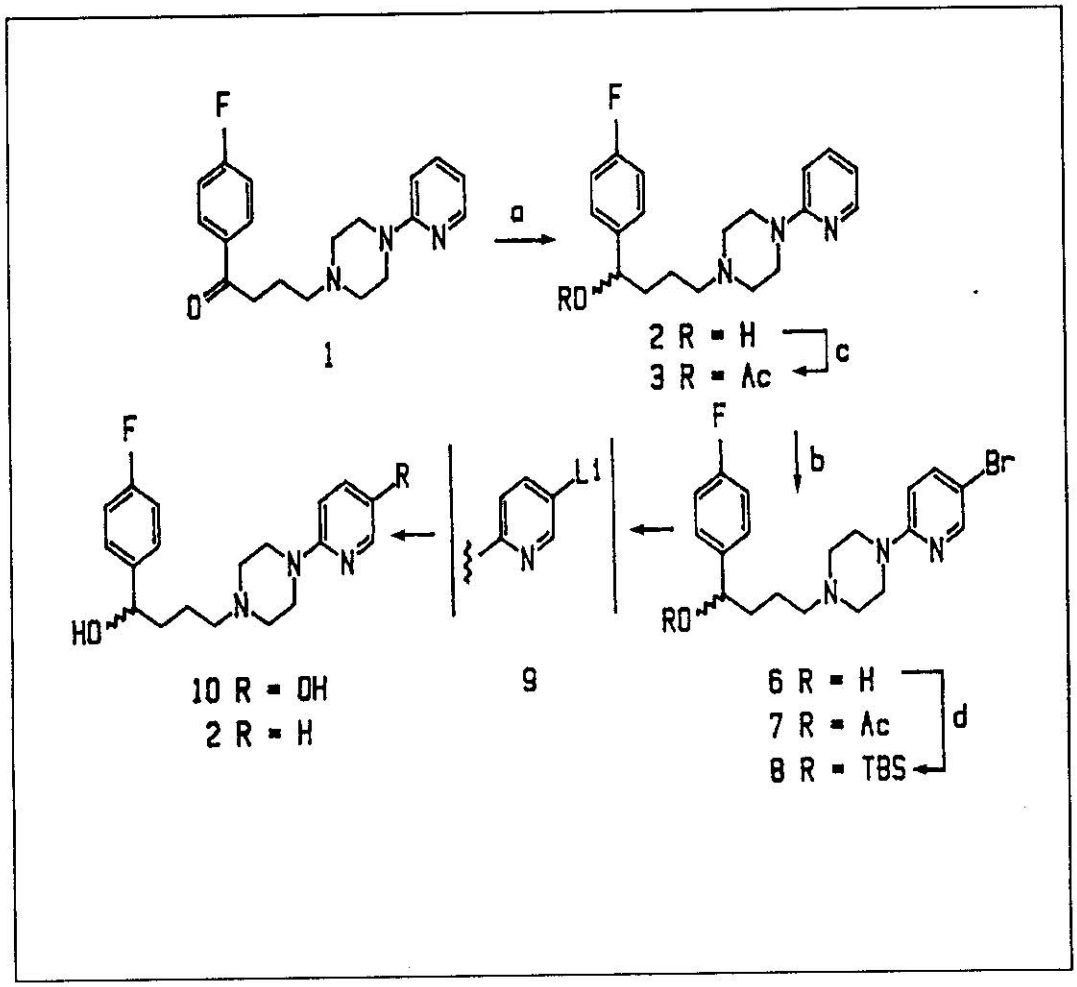
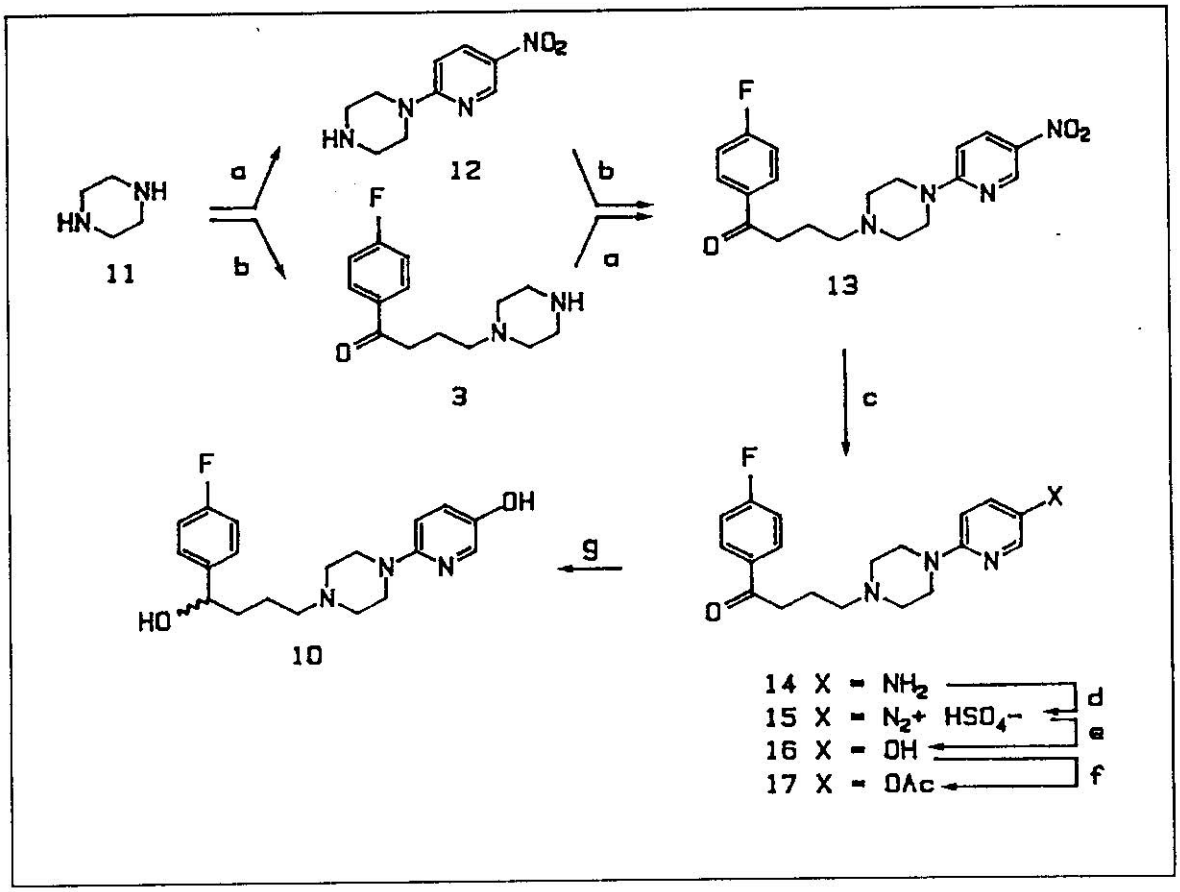


Figure 2.



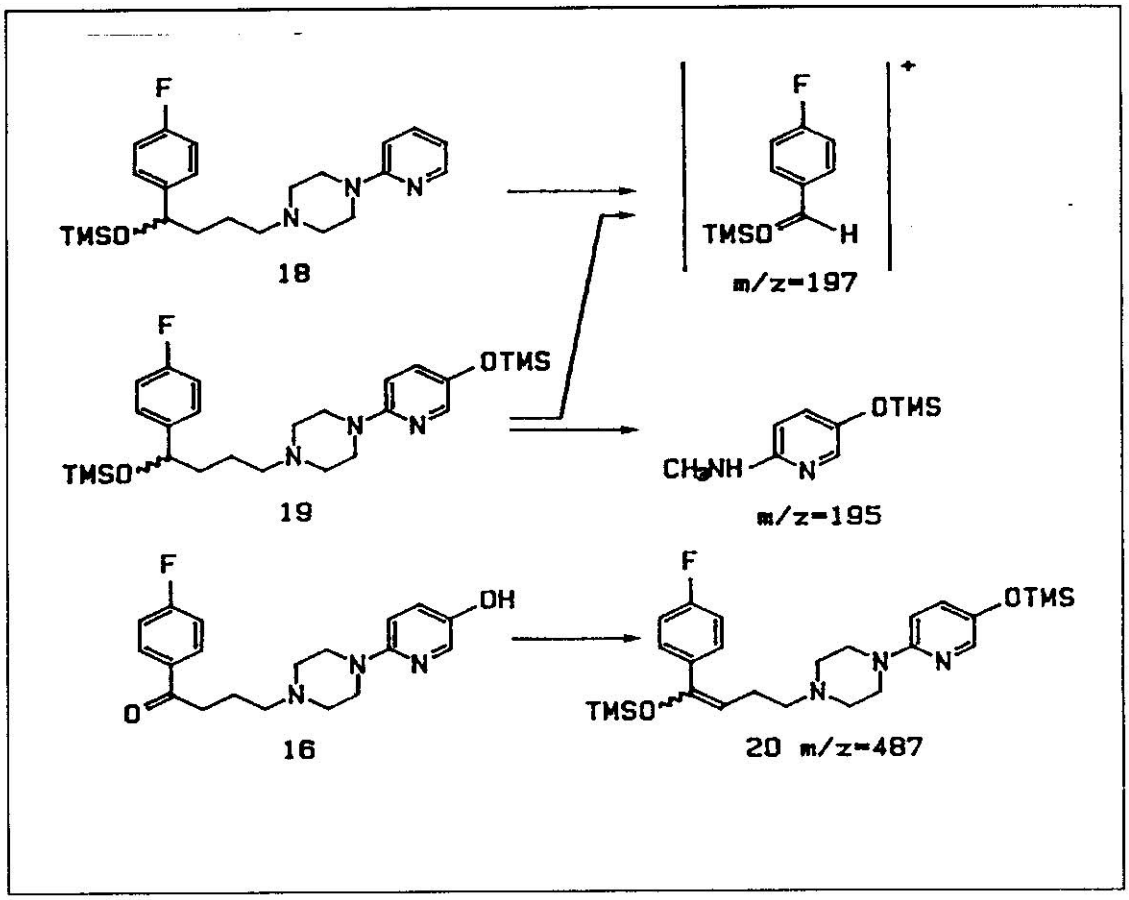
a, $LiAlH_4$, ether; b, Br_2, HOAc; c, Ac_2O, Py, DMAP; d, t-$BuMe_2SiCl$, imidazole, DMF.

Figure 3.



a, 2-bromo-4-nitropyridine, Na_2CO_3; b, Na_2CO_3, NaI, 4-chloro-1-(4-fluorophenyl)-1-butanone; c, H_2, Pd-C, MeOH; d, $NaNO_2$, H_2SO_4; e, H_3O+; f, Ac_2O, Py, DMAP; g, $LiAlH_4$, THF-ether.

Figure 4.



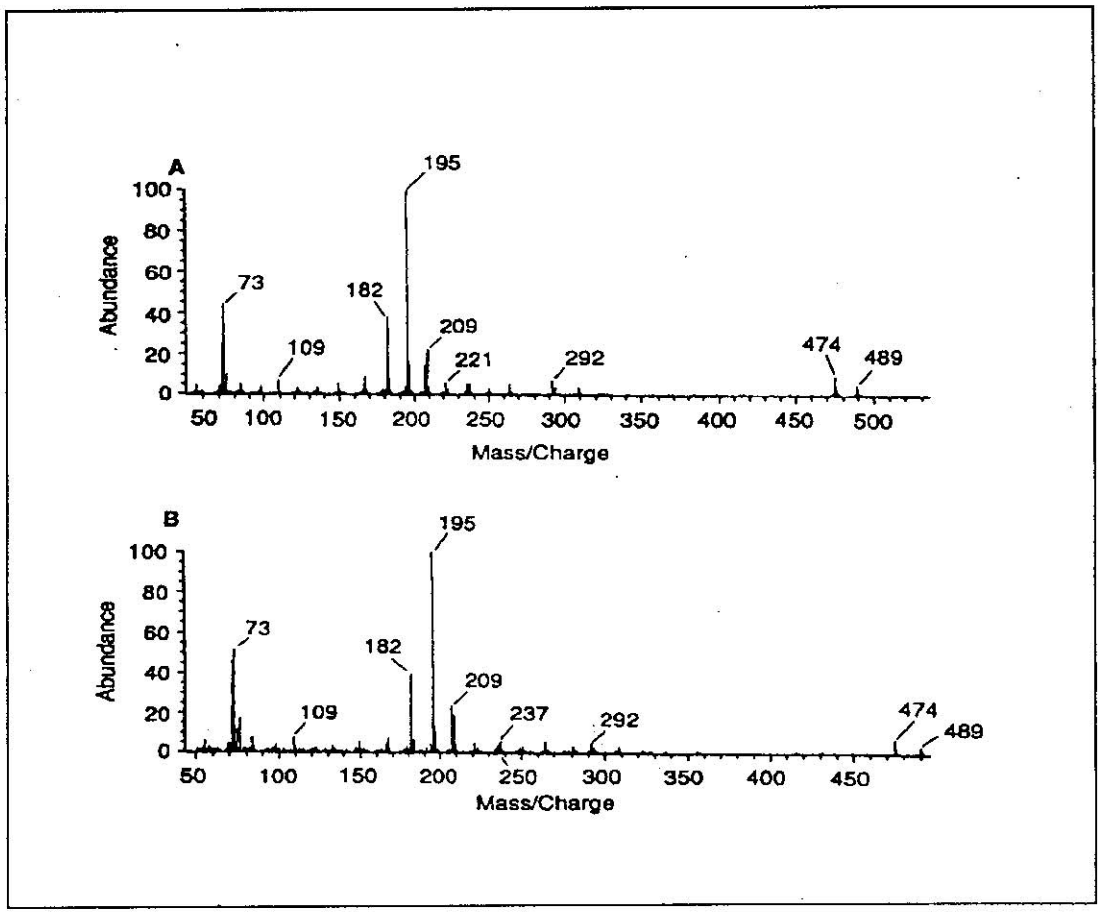


Figure 4a. Electron-impact mass spectra of the trimethylsilyl derivative of 5'-hydroxyazagerol (10) isolated from horse urine (A) and prepared by synthesis (B) an authentic standard of 5'hydroxyazaperol (10).

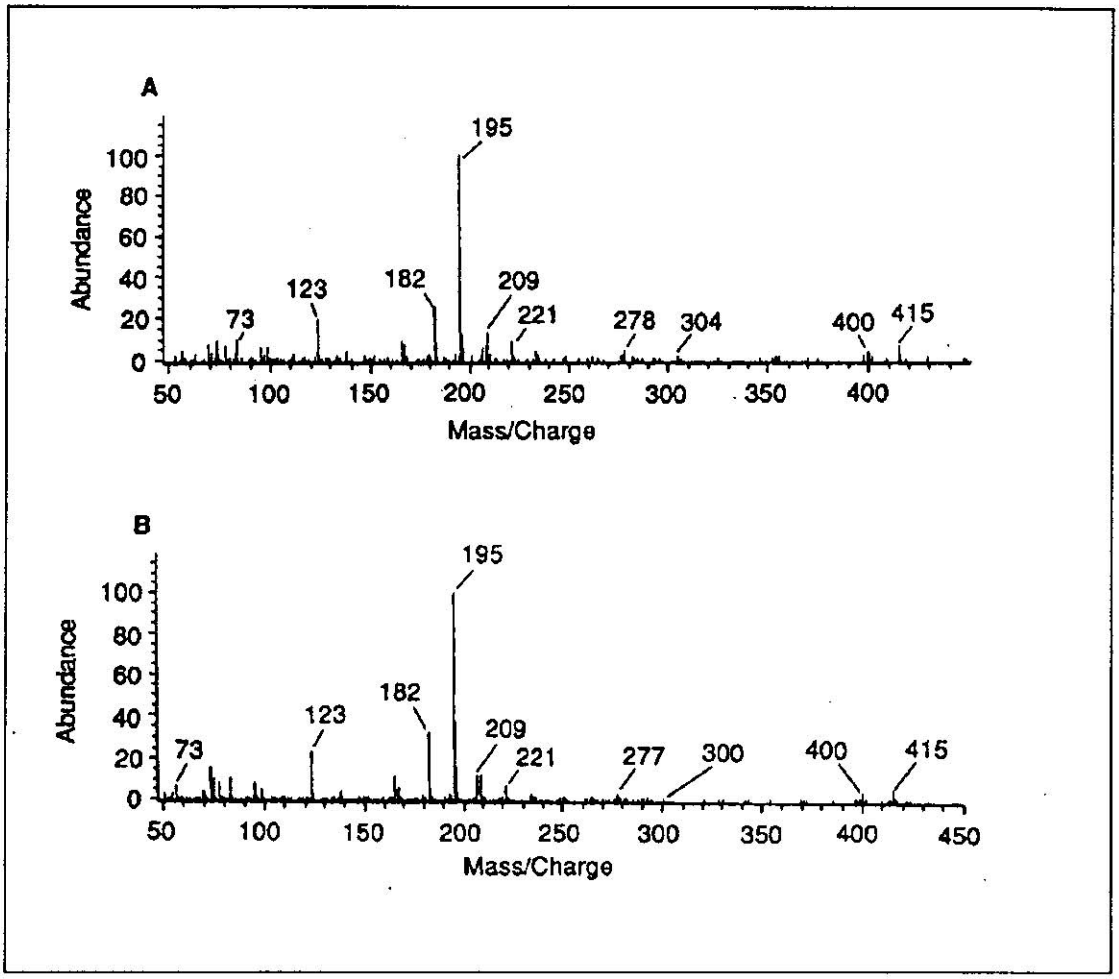


Figure 4b. Electron-impact mass spectra of the trimethylsilyl derivative of 5'-hydroxyazagerone (16) isolated from horse urine (A) and prepared by synthesis (B) an authentic standard of 5'-hydroxyazaperone.

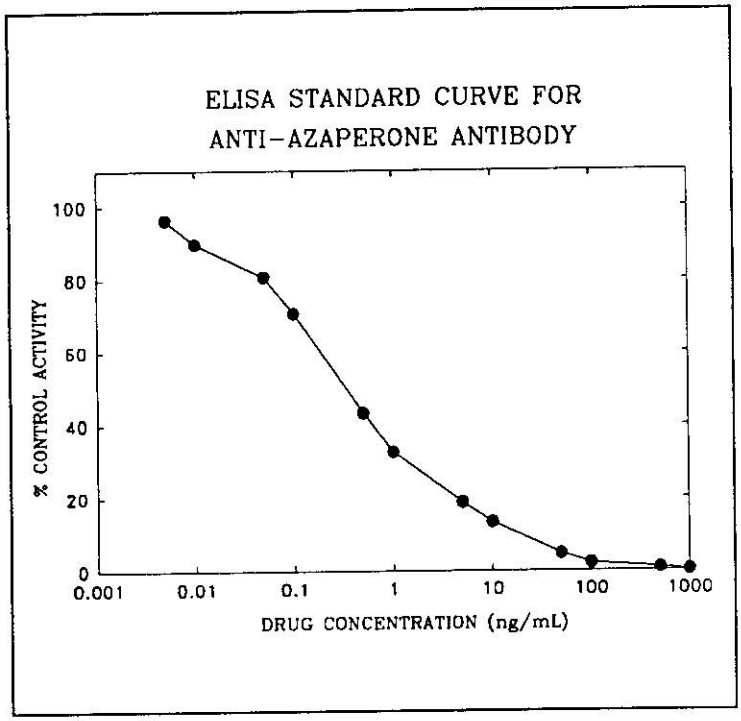


Figure 5. A standard curve for the azaperone ELISA test indicates that the addition of 400 pg/mL azaperone to the system produced about 50 percent inhibition.

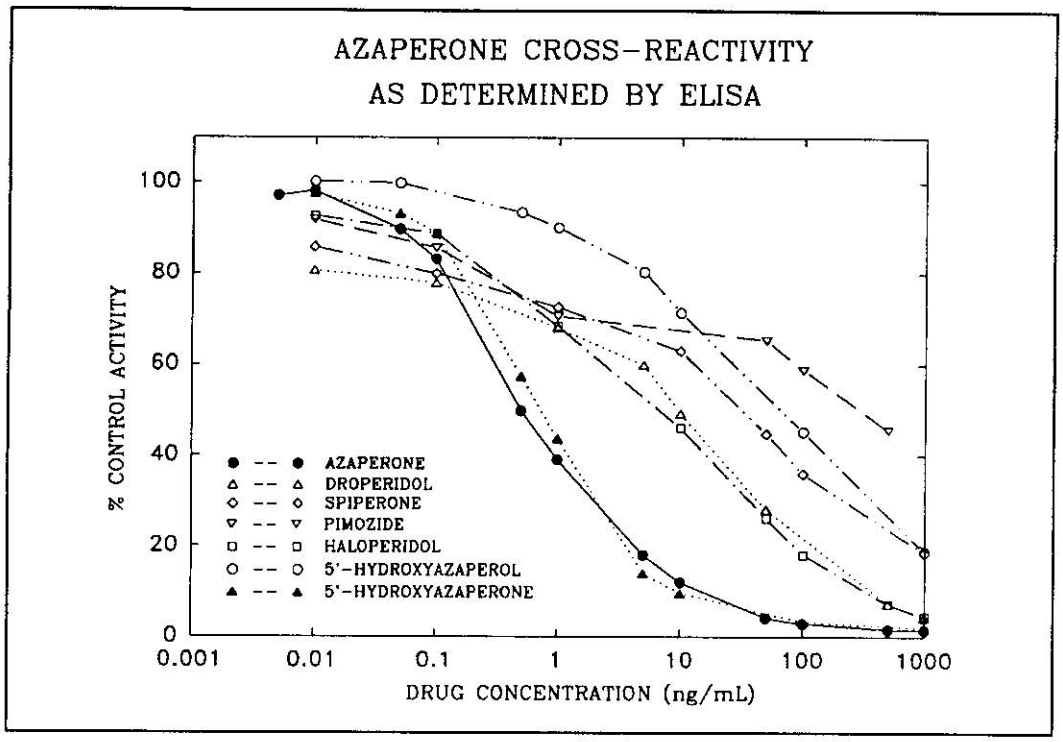


Figure 6. ELISA test activity for the anti-azaperone antibody as a function of added drug is shown. Half-maximal inhibition occurs at 0.4 ng/mL for azaperone. As listed Azaperone, Droperidol, Spiperone, Pimozide, Haloperidol, 5'-Hydroxyazaperol, 5'-Hydroxyazaperone.

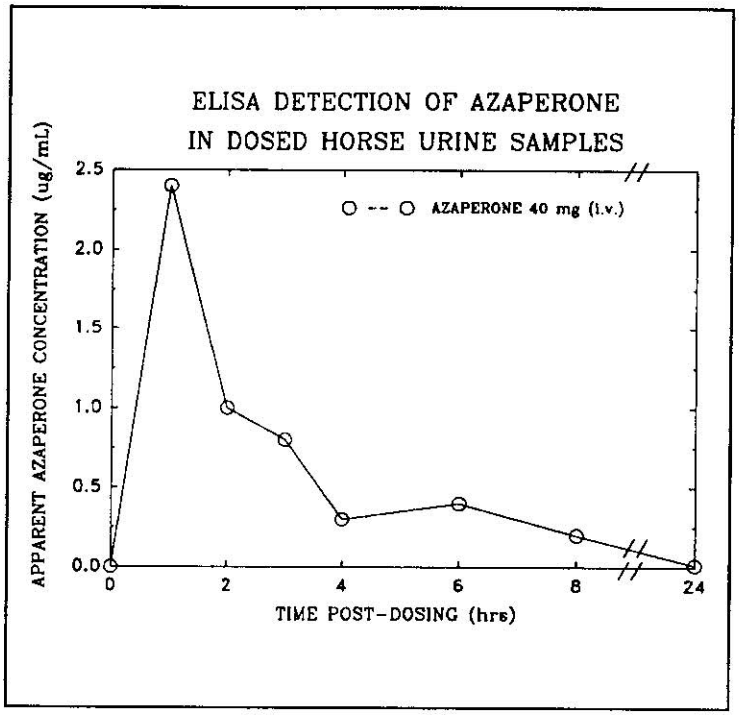


Figure 7. ELISA detection of azaperone in horse urine following a 40 mg IV dose. Control optical density was measured using the pre-dose sample.