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The Metabolism of Xylazine in Rats

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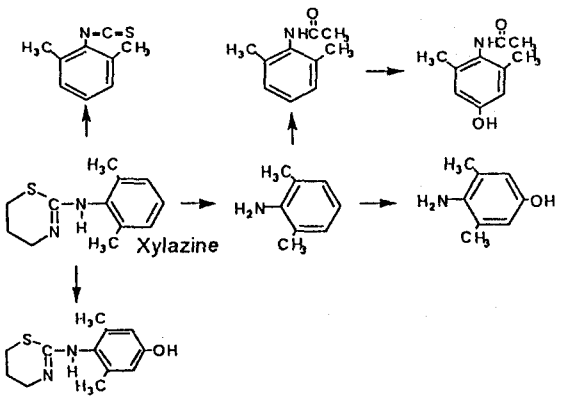
Abstract □ The biotransformation of xylazine, a widly used animal tranquilizer, was investigated in rats. After administration of xylazine, the existence of 2,6-dimethylphenyl-isothiocyanate, 2,6-dimenthyl-4-hydroxy-aniline and *p*-hydroxy-xylazine in urine was confirmed by the comparison with chemically prepared standards in GC/MS. The main metabolite, *p*-hydroxy xylazine was excreted as conjugated form.

Keywords □ Xylazine metabolism, 2,6-dimenthylphenylisothiocyanate, 2,6-dimethyl-4-hydroxy-aniline, 4-hydroxy-xylazine.

Xylazine is an animal tranquilizer effective in domestic, wild and zoo animals. In Europe, it is approved for use in cattle and commonly used to subdue cattle before delivery or slaughter. Xylazine is also a problem in horse doping because of its abused use in horse race.

The residues of xylazine in meat, milk or other biological fluids were determined by various analytical methods; They were spectrophotometric method[1], gas-chromatography[2] and high performance liquid chromatography[3]. To improve the sensitivity, sulphur-specific flame photometric detector[4] or nitrogen-specific detector[5] on the gas chromatograph are also employed. However the analysis of xylazine residues is hampered by the short excretion time and extensive metabolism of the drug. It has been reported that 70% of the radio-labelled xylazine was eliminated in urine with a half-life of about 2-3 hr, and only 8% of the activity corresponding to the unchanged form of the drug in rats[6]. Pharmacokinetic study with other animal species showed the similar results[7]. Therefore sensitive method of analysis of xylazine residue could rely on the detection of xylazine metabolite(s) rather than the drug itself. However only 2,6-dimethyl aniline is known as metabolite of xylazine[1] and other xylazine metabolites are not studied yet.

Based on the general metabolism of drugs, *p*-hydroxylation of aromatic ring, hydrolysis of 1,3-dithiazine ring and N-acetylation of aromatic amine group could be metabolic pathway of xylazine in liver. Therefore in this study, some hydrolyzed and para-hydroxylated xylazine compounds shown in Scheme 1 are chemically prepared as possible metabolites of xylazine and compared with the compounds found in urine samples by GC/MS. The synthetic procedure for the xylazine related compounds are shown in Scheme 2.

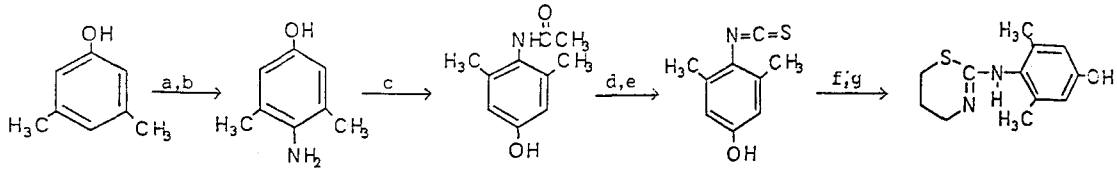


Scheme 1. Possible metabolites of xylazine.

EXPERIMENTAL METHODS

Reagents

Xylazine hydrochloride was purchased from Bayer (Seoul, Korea). 3,5-Dimethylphenol and 3-amino-1-propanol were obtained from Aldrich (Milwaukee,



Scheme 2. Synthesis of *p*-hydroxy-xylazine.
Reagents: (a) $N_2C_6H_4SO_3$, NaOH; (b) $Na_2S_2O_4$; (c) $(CH_3CO)_2O$; (d) NaH; (e) CS_2; (f) $H_2NCH_2CH_2CH_2OH$; (g) c-HCl.

WI, USA). β-Glucuronidase/aryl sulphatase (from Escherichia coli) was purchased from Boehringer (Mannheim, Germany) and Amberlite XAD-2 resin (80-150 mesh) from Serva (Westbury, NJ USA). Silica gel (70-230 mesh) used for column chromatography was obtained from Merck (Germany). Other chemicals were of analytical grade and used without purification.

Synthesis of possible metabolites

2,6-Dimethyl-4-hydroxy-aniline; The coupling of 3,5-dimethylphenol with diazotized sulfanilic acid followed by reduction with sodium dithionite as in ref. 8 gave 2,6-dimethyl-4-hydroxy-aniline; m.p. 180-181℃.

2,6-Dimethylacetanilide; 2,6-Dimethyl-4-hydroxy aniline was acetylated with acetanhydride as in ref. 9; m.p. 182-183℃.

2,6-Dimethylphenylisothiocyanate; 2,6-Dimethylacetanilide was reacted with sodium hydride followed by carbon disulfide as in ref. 10; b.p. 85℃ (0.5 mmHg).

2,6-Dimethyl-4-hydroxyphenylisothiocyanate (made according to Ref. 10 with slight modification); A rapidly stirred mixture of sodium hydride (1.6 g of a 60% dispersion, 0.04 mole) in 60 m*l* of tetrahydrofuran was warmed to 35℃ and 2,6-dimethyl-4-hydroxyacetanilide (1.79 g, 0.01 mole) was added dropwise. When the hydrogen evolution ceased, the clear reaction mixture was cooled in an ice bath and carbon disulfide (1.52 g, 0.02 mole) was added dropwise. The ice bath was then removed and the reaction mixture was stirred at room temperature for 1.5 hr. The mixture was poured into water and extracted with toluene. The organic layer was dried over sodium sulfate and the residue was subject to column chromatography using 100 g of normal phase silica. The column (2.5×60 cm) was eluted with hexane: ethylacetate (2 : 8). Evaporation of eluent solvent gave 0.86 g (48%) of solid; IR (KBr) ν 3330, 2920, 2200, 1580 cm^{-1}, ^{1}H-NMR (DMSO-d_6) δ 2.27 (s, 6H), 6.15 (s, 2H), 7.25 (s, 1H); Mass spectrum, *m/z* 146, 179 (M^+).

P-hydroxy-xylazine (synthesized by minor modification of the procedure developed for xylazine[10]): 2,6-dimethyl-4-hydroxyphenylisothiocyanate (1.00 g, 0.0056 mole) was added to 3-amino-1-propanol (0.42 g, 0.0056 mole) in 10 m*l* ether. The reaction mixture was heated to reflux for 0.5 hr. Then the solvent was evaporated *in vacuo* and 6 m*l* of concentrated hydrochloric acid was added and refluxing was continued for 1 hr. The cooled reaction mixture was treated with 60 m*l* of water and filtered. The pH of filtrate was control to 9-10 with 0.1 N sodium hydroxide and extracted with ethylacetate. After evaporation of solvent, the resulting residue was purified by chromatography on silica gel column eluted with chloroform: methanol (5 : 1) to afford 0.41 g (31%) of product as a solid; IR (KBr) ν 3350, 3300, 2940, 1580 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ 1.8 (m, 2H), 2.0 (s, 6H), 2.9 (t, 2H), 3.5 (t, 2H), 6.37 (s, 2H); Mass spectrum *m/z* 221, 236 (M^+).

Metabolism studies

Drug administrations: Xylazine hydrochloride (2% solution) was administered orally to male wistar rats (weight 200-250 g) at the dose of 80 mg/kg. The urine samples were collected to 48 hrs. The 24 hr urine samples before drug administration were also collected and used as own blank.

Extraction of xylazine related compounds in urine: Enzyme hydrolysis procedure developed by Donike *et al.*[11] was adopted. An XAD-2 slurry, previously washed with acetone, methanol and water, was filled into a Pasteur pipet upto 2.5 cm height and 5 m*l* of urine was added. The column was washed with an equal volume of water. The adsorbed lipid fraction was eluted with 3 m*l* of methanol. The metano-

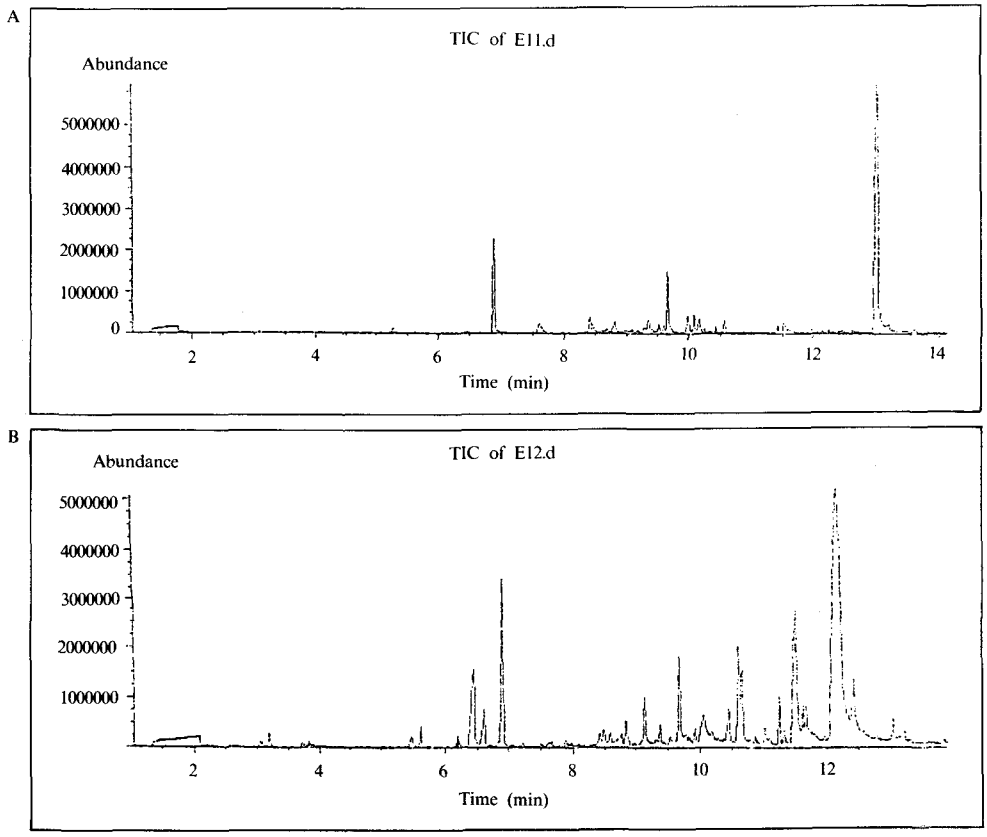


Fig. 1. Total ion chromatograms of urine extracts.
(A) Blank urine, (B) 24 hr urine.

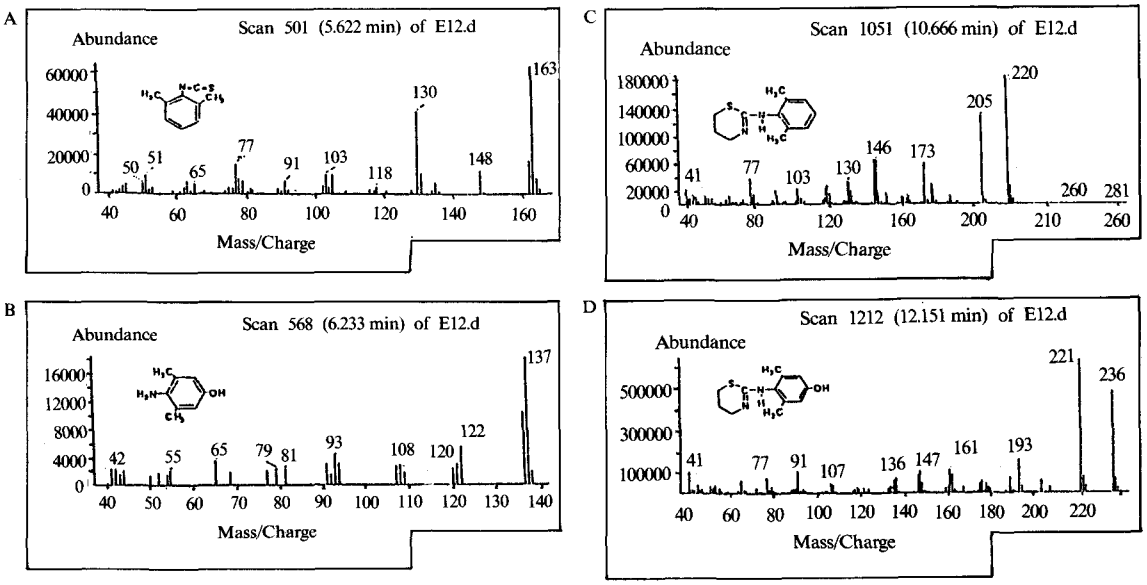


Fig. 2. Mass spectra of (A) 2,6-dimethylphenylisothiocyanate, (B) 2,6-dimethyl-4-hydroxy aniline, (C) xylazine, (D) *p*-hydroxy-xylazine.

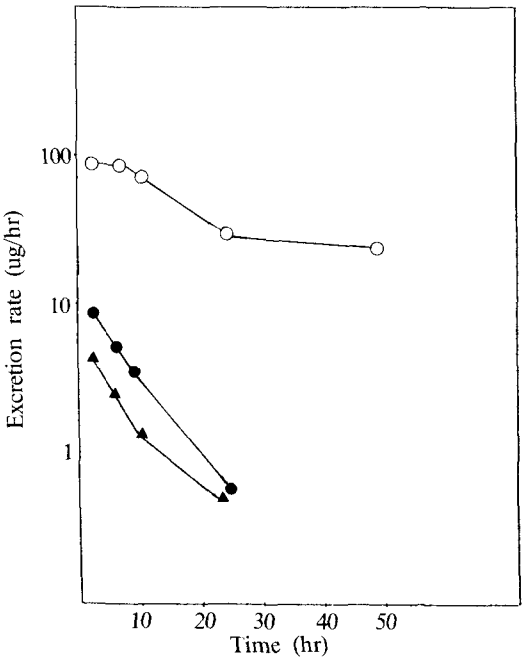


Fig. 3. Urinary excretion rate of xylazine and its metabolites.
—▲—▲— : xylazine
—●—●— : 2,6-dimethylphenylisothiocyanate
—○—○— : *p*-hydroxy-xylazine

lic solution was evaporated to dryness *in vacuo*. A 1 m*l* volume of potassium phosphate buffer (0.2 M, pH 7.0) was added to the residue, then 25 μ*l* of the enzyme preparation (β-glucuronidase; activity 10 U /m*l*, from *E. coli*) were added and the mixture was warmed to 50℃ for 1 hr.

After cooling the solution was adjusted to pH 9.6 ± 0.1 with 5 M potassium hydroxide solution containing ascorbic acid (5 m*l*: 0.5 g mixture). The sample was extracted with 8 m*l* of *n*-pentane/isopropanol (97 : 3) by mechanical shaking for 20 min. The two phases were separated by centrifugation at 1500 ×g for 5 min. The organic phase was transferred into a tube and 0.4 m*l* of 0.06 M hydrochloric acid was added and the tube was shaken mechanically for 20 min. The two phases were separated by centrifugation and the organic phase was removed by suction. The aqueous layer was dried in a desiccator (P_2O_5/KOH) and the dried residue was dissolved in 40 μ*l* of methanol and 3 μ*l* portions of the solution were injected into gas chromatograph.

Gas chromatography/mass spectrometry: Analytical gas-liquid chromatography was performed on a Hewlett-Packard 5890 instruments connected with 5970 B mass spectrometer. Analyses were performed on a closs-linked 5% phenylmethyl silicone capillary column (17 m length, 0.2 mm I.D 0.33 μm film thickness). The flow-rate of the helium was 0.70 m*l*/min. Sample was injected in split mode (split ratio; 1 : 10). Injection temperature 280℃, transferline temperature 300℃ and oven temperature, programmed from 100℃ at 20℃/min to 300℃.

RESULTS AND DISCUSSION

The GC chromatograms of the extracts from urine are shown in Fig. 1. When peaks in blank urine were substrated, several metabolite peaks were present in 24 hr urine samples from xylazine administered rats. The retention time and mass spectrum of each peak were compared with synthetic standards. The mass spectra of the identified compounds are shown in Fig. 2.

p-Hydroxy-xylazine: The major peak at 12.19 min in Fig. 1 was identified as p-hydroxy-xyazine. Its mass spectrum showed the characteristic ions *m/z* 221 and 236 (M^+), showing that the molecular weight was 16 amu higher than xylazine, which indicated the addition of an oxygen atom. This peak was also clearly detectable in 24-48 hr urines. However without β-glucuronidase hydrolysis this peak was hardly detectable, therefore it must be excreted as conjugated form. Since glucuronide conjugates are highly polar and soluble in water they are not extracted in organic solvent and not analysed in GC. So in many cases, for example detection of anabolic steroids in doping test, glucuronide conjugated metabolites are analysed after enzyme hydrolysis. When urine sample was derivatized to trimethylsilylether with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA)[12], its mass spectrum showed molecular ion at *m/z* 308.

Xylazine: The peak at 10.56 min in Fig. 1 was identified as unchanged xylazine. The mass spectrum showed ions at *m/z* 205 and 220 (M^+).

2,6-Dimethyl-4-hydroxyaniline: Very small peak at 6. 19 min in Fig. 1 was identified as 2,6-dimethyl-4-hydroxyaniline. The mass spectrum showed characteristic ions at *m/z* 122 and 137 (M^+). Hydrolysis of xylazine gives 2,6-dimethylaniline, a known metabolite of xylazine, and further hydroxylation of this compound results in 2,6-dimethyl-4-hydroxyaniline.

Table I. Relative retention times and characteristic ions of xylazine-related compoynds

Name	Metabolite of xylazine	Relative retiontion time	Characteristic ions			
2,6-dimethyl aniline	yes	0.433	77	91	106	121
2,6-dimethylphenylisothiocyante	yes	0.549	77	130	148	163
2,6-dimethyl-4-hydroxy aniline	yes	0.586	91	122	136	137
2,6-dimethyl acetanilide	no	0.625	106	120	121	163
2,6-dimethyl-4-hydroxy acetanilide	no	0.902	43	136	137	179
xylazine	yes	1	130	177	205	220
p-hydroxy-xylazine	yes	1.139	162	193	221	236

Table II. Concentration of xylazine and its metabolites in urine samples (ug/ml)

Compounds	2 hr	6 hr	10 hr	24 hr	30 hr	48 hr
xylazine	0.63	1.26	0.90	0.27	0.09	–
2,6-dimethylaniline	–	0.07	0.77	0.02	–	–
2,6-dimethylphenylisothiocyanate	0.26	2.44	1.72	0.69	0.09	–
p-hydroxy-xylazine	2.03	2.27	9.24	9.61	3.05	2.58

2,6-Dimethylphenylisothiocyanate: Unexpected metabolite of xylazine was 2,6-dimethylphenylisothioc yanate. At first this compound was prepared as synthetic intermediate for xylazine, but its retention time and mass spectrum were identical with the peak at 5.66 min in Fig. 1. The major fragment ions in the mass spectrum were at *m/z* 130, 148 and 163 (M^+). This compound was not found in the blank urine nor in the xylazine spiked urine. Isothiocyanates are known to be toxic and further study on the toxicity of this metabolite is needed since xylazine is still widely used in cattle before slaughter in Europe. The metabolic route for the formation of 2,6-dimethylphenylisothiocyanate is not known, but opening of dihydro-1,3-dithiazine ring by hydrolysis might be involved.

N-Acetylation of amino group is well known biotransformation procedure for aromatic amines, but N-acetylated 2,6-dimethylaniline was not found in xylazine administered urine samples.

In conclusion three metabolites of xylazine which have not been reported previously are identified in this study. These three metabolites were also found in xylazine administered horse urine in our lab. However application of this result on food safety test requires further investigation on food producing animal species eventhough interspecific study on the pharmacokinetics of xylazine itself showed similar results for rat, horse, cattle, sheep and dog[6,7]. The relative retention times and characteristic ions of xylazine related compounds are given in Table I.

The quantitation of these metabolites was performed on gas chromatography with nitrogen specific detection and the result is given in Table II. The concentration of unchanged xylazine was 0.1-1.3 ppm and detected up to 30 hr urine samples. The main matabolite *p*-hydroxy-xylazine showed much higher concentration up to 48 hr urine samples. Therefore detection of *p*-hydroxy-xylazine rather than xylaine is much more sensitive method in the determination of xylazine administration within 48 hr.

The excretion rates of xylazine and metabolites were plotted vs time in Fig. 3. Xylazine, 2,6-dimethylphenylisothiocyanate, and *p*-hydroxy-xylazine showed the excretion half-life of 4.5 hr, 3.6 hr, and 14.0 hr respectively. The excretion pattern of xylazine was similar to the reported result of Duhm *et al.*[6] Using radio-labelled xylazine, they found that elimination half-life was about 2-3 hr, but only 8% of the activity corresponding to the unchanged form of the drug and the presence of twenty metabolites in chromatography. Now some of the metabolites are identified in this paper. The high concentration of *p*-hydroxy-xylazine suggests that rapid elimina-

tion of xylazine is related to an intense metabolism rather than to a rapid renal excretion of unchanged xylazine.

ACKNOWLEDGEMENTS

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