Bull. Environ. Contam. Toxicol. (1992) 48:701–708

Environmental Contamination and Toxicology

Metabolic Profile of Atrazine and N-Nitrosoatrazine in Rat Urine

Giovanna Meli, Renzo Bagnati, Roberto Fanelli, Emilio Benfenati, and Luisa Airoldi

Laboratory of Environmental Pharmacology and Toxicology, Istituto di Ricerche Farmacologiche Mario Negri, Via Eritrea 62, 20157 Milano, Italy

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine, AT) is a selective triazine herbicide widely used in agriculture for the control of broadleaf weeds in many crops, including maize, sorghum and sugar cane. Because of its low chemical reactivity and solubility in water, AT is rather stable and persistent in the soil so that its residues and metabolites were found in ground water for a long time after application (Wilson et al. 1987; Bagnati et al. 1988). AT acute toxicity appears to be low in rodents (Reinhardt and Brittelli 1981); however, *in vitro* genotoxic effects have been reported after plant metabolic activation (Adler 1980). AT administered orally to rats induced DNA breaks and/or alkali-labile lesions in several organs (Pino et al. 1988); the carcinogenicity of this compound is uncertain and remains to be elucidated (Pinter et al. 1990). In the absence of conclusive data on the toxic effects of AT and because of its widespread use, reliable methods are needed for monitoring exposed populations such as agricultural workers or workers in AT chemical production as well as the general population exposed to the herbicide through contaminated drinking water.

AT is often applied on crops with nitrogen fertilizers such as nitrates which can be reduced to nitrites by microbial enzymatic activities (Kearney et al. 1977); it also contains secondary amino groups that can react with nitrite to form N-nitrosoatrazine (NAT). The formation of N-nitroso derivatives of several agricultural chemicals, including *s*-triazines, has been demonstrated (Wolfe et al. 1976; Janzowsky et al. 1980). NAT has mutagenic effects in the Ames *S. typhimurium* assay and in the Chinese hamster V-79 assay (Weisenburger et al. 1988). These observations suggest that environmental formation of NAT might be a hazard to human health. Moreover, the acidic conditions of the mammalian stomach are suitable for the endogenous synthesis of NAT from precursors ingested with the diet and drinking water (Mirvish 1975). In fact, this occurred in mice given AT and nitrite by gavage (Krull et al. 1980).

AT is reported to undergo biotransformation in mammals, N-dealkylated metabolites being the compounds formed most readily (Bakke et al. 1972; Gojmerac and Kniewald 1989). To our knowledge, no studies on NAT metabolism in mammalian systems have been reported. This paper reports comparative studies on the metabolism of AT and NAT in rats. A specific

Send reprint requests to L. Airoldi at the above address.

and sensitive gas chromatographic-mass spectrometric (GC-MS) method with selected ion recording (SIR) was developed and used for the analysis of AT, NAT and their metabolites in biological samples.

MATERIALS AND METHODS

AT was purchased from Supelco Inc., Bellefonte, PA, USA; deethyl-atrazine (2-chloro-4-amino-6-isopropylamino-*s*-triazine), deisopropyl-atrazine (2-chloro-4-ethylamino-6-amino-*s*-triazine), and diamino-atrazine (2-chloro-4,6-diamino-*s*-triazine) were obtained from Labor, Dr. Ehrenstorfer, Augsburg, Germany. Deuterated atrazine (atrazine-ethylamino-d5, AT-d5) used as external standard for quantitative analysis, was from Cambridge Isotopes Laboratories, Woburn, MA,USA. $NADP^+$, NAD^+, glucose 6-phosphate (G-6-P), G-6-P dehydrogenase (G-6-PDH) were purchased from Boehringer, Mannheim, Germany. Extrelut (large-pore kieselguhr) for liquid-liquid extraction columns, thin layer chromatographic (TLC) silica plates 60 F 0.25 mm film thickness and all the solvents used for residue analysis were obtained from Merck, Darmstadt, Germany. Reagent-grade sodium nitrite and sodium hydroxide were from Farmitalia Carlo Erba (Milan, Italy).

NAT was synthesized from atrazine and sodium nitrite as reported by Weisenburger et al. (*personal communication*). Briefly, anhydrous sodium nitrite (250 mg) was added over 2 hr to a solution of AT in glacial acetic acid (50 mg/5 ml) at room temperature. The mixture was diluted with ethyl acetate (5 ml), filtered and the pH was adjusted to 10 using 5 N NaOH. NAT was extracted from the aqueous phase with ethyl acetate (2 x 10 ml). The organic layers were combined, partially concentrated and the residual water was removed by adding anhydrous sodium sulfate. After filtration, the formation of NAT was first confirmed by TLC using dichloromethane, hexane and ethyl acetate 2 : 2 : 1 (v/v) as eluting system with UV detection. NAT was purified by preparative TLC; NAT (a yellow spot) was scraped from TLC plates and eluted with ethyl acetate. The solvent was evaporated to dryness under vacuum and the identity of NAT was confirmed by GC-MS.

Male CD rats (200 ± 10 g body weight) were purchased from Charles River (Calco, Como, Italy). The rats were housed in rooms designed to maintain approximately 22°C temperature, 50% relative humidity, with a 12-hr light cycle, for a week before use. Animals utilized in this study were acclimatized in metabolic cages for 24 hr before treatment.

Two groups of three rats were given a single oral dose of 50 mg/kg of AT or NAT dissolved in dimethylsulfoxide (DMSO). Control rats (n = 3) were treated with DMSO alone. Urine samples were collected up to 96 hr at 24 hr intervals after dosing; 100 mg of ascorbic acid were added to collection tubes to avoid *in vitro* nitrosation. Collection tubes were wrapped in aluminum foil to prevent photodegradation of N-nitroso derivatives. Urine volume was measured, the samples were centrifuged at 2500*g* and the supernatants stored frozen at -20°C until extraction.

AT, NAT and their N-dealkylated metabolites were extracted from urine as follows: 5 ml of urine were diluted to 10 ml with distilled water and the pH was adjusted to 10 with 1 N NaOH, the samples were then loaded on Extrelut columns filled with 10 g extraction phase. The compounds of interest were eluted with 20 ml of ethyl acetate and 100 ng of d5-AT were added to each sample as external standard for quantitative analysis. The eluates were

brought to dryness under vacuum and stored at -20 °C until analysis.
Recovery studies were done by adding known amounts of AT, NAT and their metabolites to urine of untreated rats and following the extraction procedure described above.
For *in vitro* experiments S9 hepatic fractions from untreated rats were used. Livers were quickly removed, minced with scissors and homogenized in 4 vols of 0.05 M phosphate buffer (pH 7.4) containing 0.15 M KCl and 0.005 M $MgCl_2$. To obtain the post-mitochondrial supernatant, the homogenate was centrifuged at 9000*g* for 20 min. Protein content was determined according to Bradford (1976). Recovery of AT, NAT and their metabolites in S9 hepatic fraction was established by adding a known amount of each compound to hepatic preparations and following the same extraction procedure. AT and NAT metabolism was studied by incubating for 30 min at 37°C, in a final volume of 1 ml, 0.9 ml of supernatant containing about 20 mg protein, the NADPH generating system (2.7 mM $NADP^+$, 50 mM G-6-P and 5 units of G-6-PDH) and 2 mM of each compound. At the end of the incubation time, AT, NAT and their metabolites were extracted as described above.
GC-MS analyses were done on a VG TS-250 mass spectrometer coupled to a gas chromatograph HP 5890. The mass spectrometer was operated in the electron impact (EI) mode with the following conditions: electron energy, 35 eV; ion source temperature, 140°C; trap current, 500 μA. GC conditions were as follows: capillary column, HP1 Methyl Sil 10 m x 0.52 mm i.d., film thickness 2.65 μm (Hewlett Packard Italiana, Cernusco sul Naviglio, Milan, Italy); oven temperature was kept at 140°C for 1 min and then programmed to 200°C at 10 °C/min followed by a second temperature ramp to 260°C at 25°C/min. The injector temperature was 140 °C, the carrier gas (He) head pressure was 5 kPa. Analyses were done in the split mode (30 ml/min).
SIR was performed by monitoring the ion intensities at m/z 215 for AT, m/z 205 for d5-AT, m/z 200 for NAT, m/z 173 for deisopropyl-atrazine, m/z 172 for deethyl-atrazine, and at m/z 145 for diamino-atrazine.
AT, NAT and their metabolites were identified in biological samples by comparison of their mass-spectra and GC retention times with the corresponding standard compounds. Total urinary mercapturic acids were measured using Ellman's reagent (Riddles et al. 1979).

RESULTS AND DISCUSSION

Mass spectra of the compounds analyzed were in agreement with previous reports (Ross and Tweedy, 1970). As shown in Table 1, the molecular ions $[M]^+$ and the ions at $[M-15]^+$, indicating the loss of a methyl group, were usually intense, thus allowing quantitative determination by GC-SIR. NAT has been reported to decompose during gas chromatographic analysis, giving AT (Wolfe et al. 1976). However under our GC conditions a single peak was detected showing a mass spectrum assigned to 2-chloro-4-(N-nitroso-N-ethylamino)-6-isopropylamino-*s* -triazine. NAT mass spectrum, besides the loss of 15, showed an intense peak at $[M-30]^+$, due to loss of the-NO group; this fragmentation is typical of nitroso compounds (Rainey et al.1978).
A typical GC-SIR chromatogram obtained by injecting a standard mixture containing 500 pg of each compound is shown in Figure 1.

Table 1. Mass spectral data of AT, NAT, and their metabolites

Compound	Fragment $[M]^+$ m/z	$[M]^+$ rel.intensity	$[M-15]^+$ m/z	$[M-15]^+$ rel.intensity
Atrazine	215	73%	200	100%
Atrazine-d5	220	65%	205	100%
N-Nitrosoatrazine	244	42%	229	20%
Deisopropylatrazine	173	100%	158	80%
Deethylatrazine	187	37%	172	100%
Diaminoatrazine	145	100%	-	-

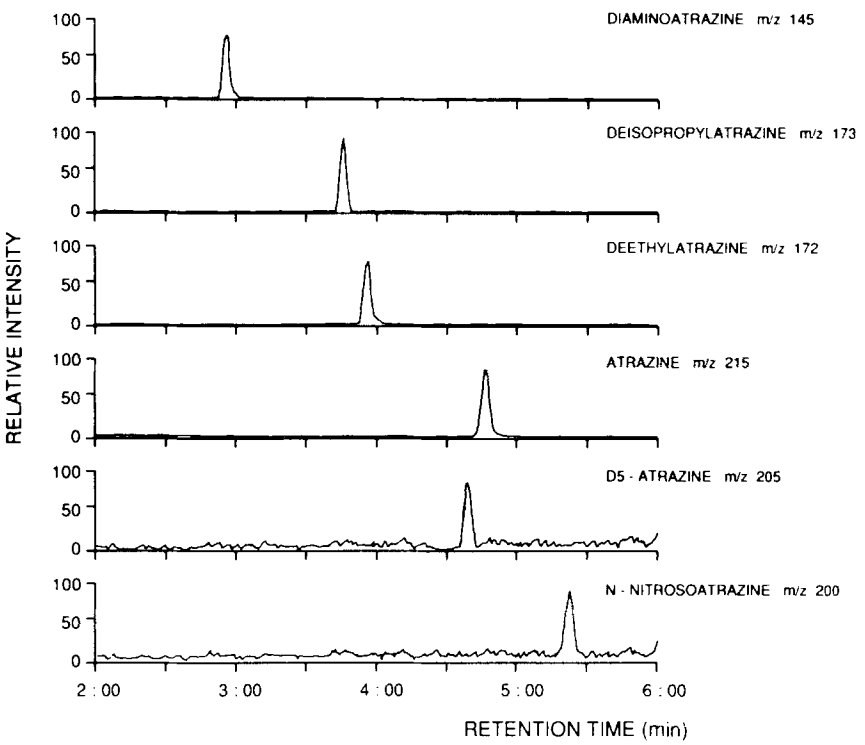

Figure 1. Typical chromatogram resulting from analysis by gas chromatography-selected ion recording of a standard mixture of atrazine, atrazine-d5, deisopropyl-atrazine, deethyl-atrazine, diamino-atrazine and N-nitrosoatrazine. Each compound was monitored at the m/z indicated.

Calibration curves obtained by analyzing increasing amounts of AT, NAT and their metabolites and a constant amount of AT-d5 showed a linear relationship between the ratio of the peak area of each compound and the

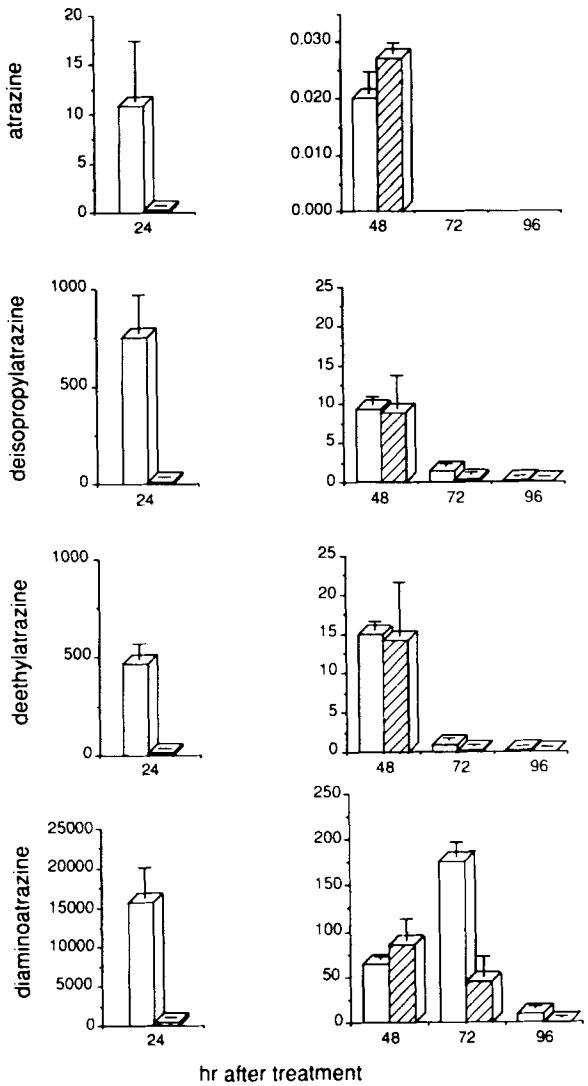

Figure 2. Metabolic profile of atrazine (white bars) and N-nitrosoatrazine (shaded bars) in urine of rats given the compounds at the dose of 50 mg/kg p.o. Bars represent nmol excreted/24 hr interval (mean ± SE of at least three urine samples).

area of AT-d5 in a range of 100-1000 pg. The correlation coefficients were 0.9974 for AT, 0.9728 for NAT, 0.9937 for deisopropyl-atrazine, 0.9912 for deethyl-atrazine, 0.9956 for diamino-atrazine. The detection limit of the method was 2 ng of each compound/sample.

Recoveries of AT, NAT, deisopropyl-atrazine, deethyl-atrazine, diamino-atrazine were 77 ± 2%, 69 ± 7%, 70 ± 4%, 91 ± 1% and 71 ± 2% respectively (mean ± SE).
Figure 2 shows the urinary excretion profile after oral AT and NAT (50 mg/kg) in the rat. After AT, the compound was readily converted into metabolites resulting from N-dealkylation. The formation of these metabolites has been reported both *in vivo* and *in vitro* (Bakke et al. 1972; Gojmerac and Kniewald 1989; Foster et al. 1979). The urinary excretion of AT and its metabolites was highest at 24 hr and decreased steeply; unchanged AT was not detected for more than 48 hr after dosing. The urinary excretion pattern of NAT in the rat indicated that a small fraction undergoes N-denitrosation to AT, this then being metabolized to the same N-dealkylated products as in the urine of rats given AT. No unchanged NAT was detectable in urine.
Table 2 shows the percentage of urinary excretion of each metabolite during the 0-96 hr interval after dosing.

Table 2. 0-96 hr Overall urinary excretion of AT, NAT and their metabolites: percentages of administered dose

Compound Excreted	Compound Administered	
	AT	NAT
Atrazine	0.03 ± 0.01	0.001 ± 0.0001
Deisopropylatrazine	1.64 ± 0.42	0.05 ± 0.01
Deethylatrazine	1.03 ± 0.17	0.06 ± 0.02
Diaminoatrazine	34.26 ± 8.32	1.46 ± 0.11
TOTAL	36.95 ± 8.89	1.57 ± 0.12

Values are means ± SE of at least 3 rats/group.
AT or NAT were administered at the dose of 50 mg/kg p.o.

In agreement with previous data, the urine appears to be the principal route of elimination of AT in rats (Bakke et al. 1972; Timchalk et al.1990). Overall urinary excretion of AT and its metabolites in rats given AT was approximately 37% of the administered dose, while the total excretion of the group dosed with NAT was less than 2%. The presence of a nitroso moiety in the atrazine molecule at the amino group bearing the ethyl chain seems to influence the metabolic fate of NAT, the total amount of metabolites and parent compound excreted in the urine of rats given NAT being about 20 times less than after AT.
Urinary excretion of mercapturic acids has been reported in rats given s-triazinic compounds (Timchalk et al. 1990). In order to clarify the different urinary excretion of AT and NAT, we investigated conjugation with glutathione. The excretion of mercapturic acids was measured in urine of rats given AT or NAT.
Although the total amount of mercapturic acids appeared to be higher in urine of treated rats than in controls, the difference was not significant (data not shown). Thus conjugation with glutathione and mercapturic acid

excretion do not appear to be the main route of elimination of NAT and cannot explain the difference between NAT and AT metabolism observed in this study. The formation of s-triazine hydroxy-derivatives has been reported (Bakke et al. 1972; Gojmerac and Kniewald 1989), suggesting that conjugation with glucuronic acid might occur. Enzymatic digestion with β-glucuronidase-arylsulfatase on urine samples did not reveal the presence of glucuronides (data not shown).

A final attempt was made to investigate the metabolic fate of NAT by analyzing urine samples by GC coupled to a detector specific for N-nitroso compounds, the thermal energy analyzer. No N-nitroso compound was detected at any time (data not shown).

As shown in Table 3, *in vitro* experiments confirmed the different rate of metabolism of AT and NAT observed *in vivo*. These results suggest that a different biodegradation pathway for NAT might exist in mammals and this is currently under investigation in our laboratory.

Table 3. *In vitro* biotransformation of AT and NAT by hepatic S9 fractions: % of amount incubated

Compound Detected	Compound Incubated	
	AT	NAT
N-Nitrosoatrazine	-	7.41 ± 0.001
Atrazine	44.68 ± 10.05	24.04 ± 0.94
Deisopropylatrazine	29.16 ± 4.08	5.18 ± 0.21
Deethylatrazine	7.65 ± 1.63	2.39 ± 0.15
Diaminoatrazine	0.68 ± 0.14	n.d.
TOTAL	82.17 ± 14.38	39.03 ± 0.75

Values are means ± SE for 3 samples/group.
AT or NAT (2mM) were incubated with hepatic S9 at 37°C for 30 min.

The fully dealkylated metabolite diamino-atrazine was the major metabolite formed *in vivo* after AT and NAT, suggesting that cytochrome P-450-dependent N-dealkylation occurred promptly. This metabolite is readily excreted and recovered in urine in high levels; it can be well quantified for a long time after dosing. Thus, diamino-atrazine in urine could be used for a quantitative assessment of exposure to triazinic compounds. The highly specific and sensitive method described in this paper seems to be useful for studies on human exposure to s-triazines.

Acknowledgments. We thank Ms. J. Baggott and the staff of the G.A. Pfeiffer Memorial Library who helped prepare the manuscript. This work was supported by the Italian National Research Council, contract No. 90.00637.CT13.

REFERENCES

Adler ID (1980) A review of the coordinated research effort on the comparison of test systems for the detection of mutagenic effects, sponsored by the E.E.C. Mutat. Res. 74:77-93

Bagnati R, Benfenati E, Davoli E, Fanelli R (1988) Screening of 21 pesticides in water by single extraction with C18 silica bonded phase columns and HRGC-MS. Chemosphere 17:59-65

Bakke JE, Larson JD, Price CE (1972) Metabolism of atrazine and 2-hydroxyatrazine by the rat. J. Agric. Food Chem. 20:602-607

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254

Foster TS, Khan SU, Akhtar MH (1979) Metabolism of atrazine by the soluble fraction (105000g) from chicken liver homogenates. J. Agric. Food Chem. 27: 300-303

Gojmerac T, Kniewald J (1989) Atrazine biodegradation in rats- A model for mammalian metabolism. Bull. Environ. Contam. Toxicol. 43:199-206

Janzowsky C, Klein R, Preussmann R (1980) Formation of N-nitroso compounds of the pesticides atrazine, simazine and carbaryl with nitrogen oxides. In: Walker EA, Castegnaro M, Griciute L, Borzsonyi M (Eds) N-Nitroso Compounds: Analysis, Formation and Occurrence, IARC Scientific Publications no. 31. IARC, Lyon, p 329-339

Kearney PC, Oliver JE, Helling CS, Isensee AR, Kontson A (1977) Distribution, movement, persistence and metabolism of N-nitrosoatrazine in soils and a model aquatic ecosystem. J. Agric. Food Chem. 25:1177-1181

Krull IS, Mills K, Hoffman G, Fine DH (1980) The analysis of N-nitrosoatrazine and N-nitrosocarbaryl in whole mice. J. Anal. Toxicol. 4:260-262

Mirvish SS (1975) Formation of N-nitroso compounds: chemistry, kinetics, and in vivo occurrence. Toxicol. Appl. Pharmacol. 31:325-351

Pino A, Maura A, Grillo P (1988) DNA damage in stomach, kidney, liver and lung of rats treated with atrazine. Mutat. Res. 209:145-147

Pinter A, Török G, Börzsönyi M, Surjan A, Csik M, Kelecsenyi Z, Kocsis Z (1990) Long-term carcinogenicity bioassay of the herbicide atrazine in F344 rats. Neoplasma 37:533-544

Rainey WT, Christie WH, Lijinsky W (1978) Mass spectrometry of N-nitrosamines. Biomed. Mass Spectrom. 5:395-408

Reinhardt CF, Brittelli MR (1981) Heterocyclic and miscellaneous nitrogen compounds. In: Clayton GD, Clayton FE (Eds) Patty's Industrial Hygiene and Toxicology, 3rd rev. ed., vol 2A. John Wiley and Sons, New York, p 2671-2822

Riddles PW, Blakeley RL, Zerner B (1979) Ellman's reagent : 5,5' dithiobis(2-nitrobenzoic acid) - a reexamination. Anal. Biochem. 94:75-81

Ross JA, Tweedy BG (1970) Mass spectra of chloro-,aminochloro- and ethylaminochloro triazines. Organic Mass Spectrometry 3:219-229

Timchalk C, Dryzga MD, Langvardt PW, Kastl P.E., Osborne DW (1990) Determination of the effect of tridiphane on the pharmacokinetics of [14 C]-atrazine following oral administration to male fischer 344 rats. Toxicology 61:27-40

Weisenburger DD, Joshi SS, Hickman TI, Walker BA, Lawson TA (1988) Mutagenesis tests of atrazine and N-nitrosoatrazine. Compounds of special interest to the Midwest. Proc. Am. Ass. Cancer Res. 29:106

Wilson MP, Savage EP, Adrian DD, Aaronson MJ, Keefe TJ, Hamar DH, Tessari JT (1987) Groundwater transport of the herbicide, atrazine, Weld County, Colorado. Bull. Environ. Contam. Toxicol. 39:807-814

Wolfe NL, Zepp RG, Gordon JA, Fincher RC (1976) N-nitrosamine formation from atrazine. Bull. Environ. Contam. Toxicol. 15:342-347

Received September 20, 1991; accepted December 2, 1991.