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Short communication

Identification of a metabolite of atrazine, *N*-ethyl-6-methoxy-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine, upon incubation with rat liver microsomes

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Abstract

Atrazine is an herbicide which has shown potential antimalarial effects both in vitro and in vivo in rats. In order to study the metabolism of atrazine in rat livers, we developed a sensitive LC/MS/MS method for the identification of atrazine and several of its metabolites. Using this method, we identified one previously unreported metabolite with a mass of 211 Da in addition to two known metabolites. This new metabolite was confirmed to be *N*-ethyl-6-methoxy-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine, also known as atraton, by comparison of the LC/MS/MS mass spectra and the retention time to those of a commercial standard.

Keywords: Atrazine; Atraton; LC/MS/MS; Metabolite

1. Introduction

Atrazine, 6-chloro-*N*-ethyl-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine (see Fig. 1) is an herbicide used world-wide and it is one of the most heavily used agricultural herbicides in North America [1]. Many efforts have been made to measure atrazine and its metabolites in environmental samples, such as water, soil, and vegetables in order to assess its potential toxicity. Kovacic et al. determined atrazine metabolites in soil using HPLC with photodiode array detection coupled with microwave-assisted solvent extraction [2]. Yolkey and Cheung reported a GC/MS method for detecting atrazine and its dealkylated chlorotriazine metabolites in water [3]. In human urine, atrazine and its metabolites can be detected by LC/MS/MS with Atmosphere Pressure Chemical Ionization [4] or electrospray ionization (ESI) [5]. With the application of solid phase extraction (SPE), quantification limits of atrazine can be as low as 0.5 ng/L [6]. Mendas et al. analyzed atrazine and three chlorodealkylated atrazine metabolites from human urine using capillary GC with nitrogen selective and electron capture detectors [7].

A plastid organelle, the apicoplast, is found in apicomplexan parasites, such as the malarial parasite. Studies have shown that the apicoplast is a degenerate chloroplast [8]. While the apicoplast cannot photosynthesize, it is essential for the survival of malaria parasites. Thus, it is a new antimalarial drug target and herbicides which can affect chloroplasts may also act as antimalarial agents [9]. Previous research in our group has shown that atrazine has potential antimalarial effects both in vitro and in vivo in rats (submitted for publication in American Journal of Tropical Medicine and Hygiene).

In this research, we developed a LC/MS/MS method, which can be used to measure atrazine and its major metabolite in rat hepatic microsomal samples. We identified a previously unreported metabolite of atrazine, *N*-ethyl-6-methoxy-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine (see Fig. 1), using this method.

Abbreviations: SIM, single ion monitoring; SPE, solid phase extraction; CID, collision-induced dissociation; ESI, electrospray ionization

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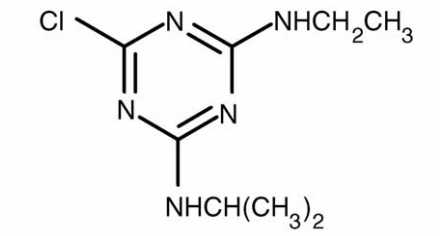
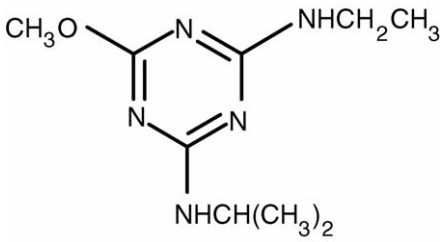


Atrazine MW: 215



Metabolite MW: 211

Fig. 1. Structure of atrazine and metabolite.

2. Experimental

2.1. Chemicals

Atrazine (97.2%) was purchased from Supelco (Bellefonte, PA). Atraton (95.4%), desethylatrazine (99.9%), and desisopropylatrazine (96.3%) were purchased from the Sigma–Aldrich Chemical Co. (St. Louis, MO). Acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ); the water was double distilled, deionized, and 0.2 μm filtered (Nanopure, Barnstead, IA).

2.2. Preparation of rat liver microsomes

Female Sprague-Dawley rats aged approximately 50 days and weighing 180–250 g were housed in plastic cages at 22–26 °C and 50–60% humidity under a 12-h light/dark cycle for 1 week. The animals were decapitated and the livers were quickly removed, perfused with cold saline and stored at −80 °C. Rat liver microsomes were prepared as described by Rettie et al. [10]. Briefly, the liver samples were thawed at room temperature, then homogenized in a hand-held Teflon-glass homogenizer in three volumes of homogenization buffer, which contained 0.25 mmol/L sucrose, 1 mmol/L EDTA and 50 mmol/L potassium phosphate buffer at pH 7.4. The homogenized tissue was centrifuged at 13,500 × *g* for 20 min. The supernatant was then centrifuged at 105,000 × *g* for 60 min. The microsomal pellet was resuspended in homogenization buffer and centrifuged at 105,000 × *g* for 60 min again. The final microsomal pellet was stored at −80 °C in buffer containing 100 mmol/L potassium phosphate, 20% glycerol, 1 mmol/L EDTA at pH 7.4. The protein concentration of microsomes was measured with the BCA™ Protein Assay Kit (PIERCE, Rockford, IL).

2.3. In vitro incubation with rat liver microsomes

Atrazine was incubated with rat liver microsomes. A typical incubation mixture consisted of 10 μL of substrate (25–100 μmol/L), 150 μL liver microsomes with a protein concentration of 8.2 mg/mL, 100 μL NADPH (10 mmol/L), and 100 mmol/L potassium phosphate buffer (pH 7.4), with a final volume of 1 mL. The reaction was initiated by addition of the NADPH. After incubation at 37 °C for 20 min, the reaction was terminated by adding 4 mL ethyl acetate. The mixture was then vigorously extracted for 2 min by vortexing, and then centrifuged at 1000 × *g* for 10 min. The organic fraction was evaporated under a gentle steam of N_2 at room temperature. The residue was dissolved in 1 mL of methanol for further preparation by SPE.

2.4. Solid phase extraction

The methanolic residues were extracted using a mixed-mode cation exchange SPE cartridge (OASIS® MCX, 3 cm^3, 75 mg, Waters, Millford, MA). Samples were adjusted to pH 1.5 with 0.1 mol/L HCl. The cartridge was conditioned with 1 mL methanol followed by 1 mL water. After loading 1 mL of the sample, the SPE cartridge was washed first with 1 mL 0.1 mol/L HCl and then 1 mL methanol. The analytes were then eluted with 1.5 mL 4% NH_4OH–0.1 mol/L HCl. The eluents were then gently evaporated under a N_2 stream at room temperature. The residues were dissolved in 200 μL acetonitrile aliquots and analyzed by LC/MS/MS.

2.5. LC/MS/MS analysis

LC/MS/MS analysis was carried out with a ThermoFinnigan LCQ Advantage Quadruple Ion Trap Mass Spectrometer with Xcalibur® version 1.4 software interfaced to a Waters 2795 HPLC system. Chromatography was performed by gradient elution on a Luna C_{18} (2) column (4.6 mm × 150 mm, 5 μm, Phenomenex, Torrence, CA). Aliquots of 5 μL were injected and eluted at a flow rate of 0.5 mL/min. The column temperature was maintained at 30 ± 1 °C. The mobile phase consisted of 0.1% formic acid in acetonitrile (solvent A) and 0.1% formic acid in water (solvent B) delivered by a nonlinear gradient (convex curve 5 of the Waters HPLC system) from 30 to 70% solvent A over a 20 min period. Mass spectrometry experiments were performed with ESI in positive ion mode. The capillary voltage was fixed at 46 V, and its temperature was maintained at 350 °C. The spray voltage was set at 2.0 kV. The HPLC column effluent was nebulized using N_2 as both the sheath and auxiliary gas at flow rates of 63 and 35 units, respectively (units specific to ThermoFinnigan systems). The MS/MS spectra were produced by collision-induced dissociation (CID) of the selected precursor ions with He, and the relative collision energy was set at 35% (units specific to ThermoFinnigan systems). Both full scan and single ion monitoring (SIM) modes were used in order to detect the metabolites. SIM mode target ions of 174, 188 and 232 *m/z* were selected as these represented likely known metabolites, as well as 216 *m/z* (atrazine). Extracted ion chromatograms

yielded an unexpected peak arising from an ion at 212 *m/z* and thus SIM chromatograms at 212 *m/z* were also taken. Ion chromatograms were generated by monitoring the above ions (each on a separate chromatographic injection) for the reconstituted SPE residues containing atrazine. Control samples containing no atrazine were similarly analyzed.

3. Results and discussion

A previously unreported metabolite of atrazine was identified. Fig. 2 shows a representative full scan ion chromatogram from 100 to 250 *m/z* of the reconstituted SPE residues containing atrazine. Atrazine appears at t_R 17.3 min and the new metabolite at t_R 7.6 min. The mass spectrum of the t_R 7.6 min peak gave a base peak with *m/z* of 212 (Fig. 3-top panel). This ion was assigned as the singly protonated precusor of the new metabolite after analysis of the carbon isotopic ratios.

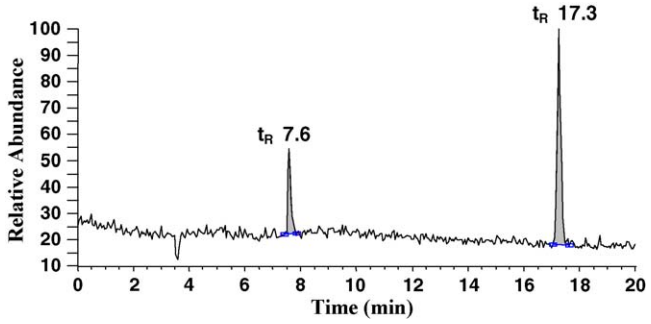


Fig. 2. Ion chromatogram of atrazine-containing SPE residue, scanning 100–250 *m/z*. The peak at t_R 7.6 min is the new metabolite; the peak at t_R 17.3 min is atrazine.

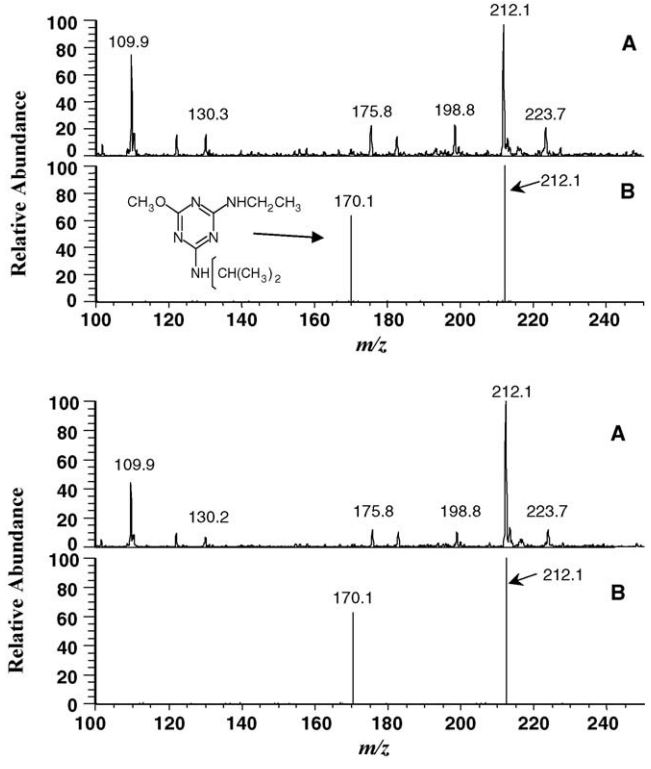


Fig. 3. Comparison of the mass spectra of metabolite in a reconstituted SPE residues (top panel) and atraton standard (bottom panel). The metabolite shows a fragmentation pattern identical to that of the atraton standard at a 35% collision energy. A shows the mass spectrum from 100 to 250 *m/z* of the peak at t_R 7.6 min and B shows the fragments of the 212 *m/z* ion.

The SIM chromatograms of the reconstituted SPE residues containing atrazine yielded prominent peaks at 7.6 min (212 *m/z*), 8.6 min (174 *m/z*), 10.6 min (188 *m/z*), and 17.3 min (atrazine). No such chromatographic peaks were observed in the control samples. The peaks at 8.6 and 10.6 min were assigned as previously reported metabolites of atrazine, desisopropylatrazine and desethylatrazine, respectively, in rats [11]. The ion with 212 *m/z* has not been previously reported in association with the metabolism of atrazine.

Subsequent CID of the ion at 212 *m/z* almost exclusively produced a product ion at 170 *m/z*, which indicated a fragment loss of 42 Da. The CID of atrazine produced an ion at 174 *m/z*, which also demonstrated a fragment loss of 42 Da. This is consistent with the loss of an isopropyl group (see Fig. 3).

The new metabolite (MH^+) is detected at 212 *m/z*. A commercial atrazine analog, atraton, has a molar mass of 211 Da. Comparison of the SIM ion chromatograms (data not shown) and MS/MS spectra for the atraton standard and the new metabolite indicate that they are identical; the t_R of both was 7.6 min with nearly identical MS/MS spectra (see Fig. 3).

4. Conclusion

Previously, one major metabolite of atrazine, desisopropylatrazine and two minor metabolites, desethylatrazine, and 1-hydroxyisopropylatrazine have been identified in male and female rats [11]. In this research, we detected the former two, but not the latter. We did, nevertheless, identify a new metabolite, *N*-ethyl-6-methoxy-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine, also known as atraton (see Fig. 1). Atraton is an herbicide and may also have antimalarial effects. If this is true, the metabolism of atrazine may have significant implications for its antimalarial effects. Clearly, atrazine can be metabolized into several metabolites and further investigation is needed.

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