Quantitative Identification of Atrazine and its Chlorinated Metabolites in Plasma

Jill M. Brzezicki, Melvin E. Andersen, Brian K. Cranmer, and **John D. Tessari**
Department of Environmental and Radiological Health Sciences, Colorado State University, Ft. Collins, Colorado 80523

Abstract

The objective of this study was to develop an analytical method to detect and quantitate the chlorotriazine herbicide atrazine (ATRA), and its chlorinated metabolites [desethylatrazine (DE-ATRA), desisopropylatrazine (DI-ATRA), and diaminochlorotriazine (DACT)] in plasma. Control plasma separated from whole rat blood was fortified with known concentrations of ATRA, DE-ATRA, DI-ATRA, and DACT. These compounds were extracted from the plasma using a liquid–liquid extraction technique, and the resulting extracts were derivatized with tetrabutyl ammonium hydroxide and methyl iodide to produce methylated derivatives of ATRA and its chlorinated metabolites. Derivatized samples and standards were analyzed using gas chromatography–mass spectrometry with selected ion monitoring. Recoveries of fortified plasma samples ranged from 84% to 97% and were validated to 100 ng/mL. This analytical method was subsequently verified in a small-scale animal study to determine time course concentrations of chlorotriazines in plasma following a single oral gavage dose of ATRA to female Sprague Dawley rats.

Introduction

Atrazine (ATRA, 2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a commonly used triazine herbicide applied as a ground or aerial spray to corn, sorghum, sugarcane, Christmas trees, and other crops to control broadleaf weeds. ATRA is a U.S. Environmental Protection Agency (EPA) restricted-use herbicide because of its potential for contaminating groundwater. ATRA moves readily through soil, with both ATRA and its dealkylated degradation byproducts often detected in surface and ground water (1). ATRA metabolism in vivo occurs primarily through cytochrome P450 heteroatom *N*-dealkylation and subsequent glutathione conjugation resulting in nonchlorinated conjugates that are excreted in the urine (Figure 1).

The chlorinated triazines (including metabolites) have been shown to cause adverse neuroendocrine and reproductive effects at high doses. Lifetime exposure to ATRA increased the prevalence and decreased the latency of mammary tumor formation in female Sprague Dawley rats by altering the neuroendocrine hormonal balance. At high doses, ATRA suppresses the estrogen-induced leutinizing hormone (LH) surge and prolactin release in ovariectomized rats (2). The target site of action has been reported to occur through the hypothalamus by increased dopamine and decreased norepinephrine concentrations in the hypothalamus (2). ATRA has been shown to delay the onset of puberty and alter estrous cyclicity in female Wistar rats (3).

The U.S. EPA has classified ATRA as a possible (Group C) human carcinogen (4), but it has not classified it as a definitive carcinogen because of limited animal evidence in the absence of human data (5). Human carcinogenicity assessments of ATRA have been based on epidemiology studies relating ATRA (and other triazine) exposure to leukemia, ovarian cancer, non-Hodgkin's lymphoma, Hodgkin's disease, multiple myeloma,

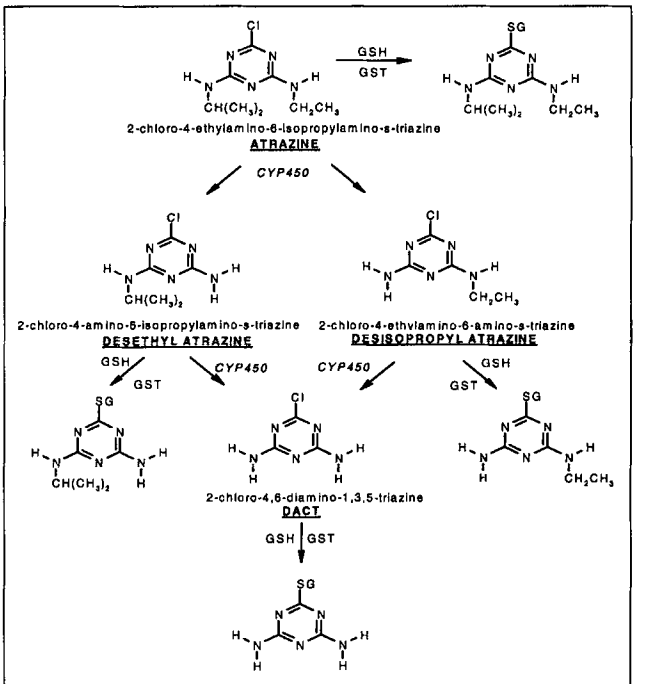

Figure 1. Metabolic pathway of ATRA following P450 heteroatom *N*-dealkylation and glutathione conjugation.

* Author to whom correspondence should be addressed. E-mail: jbrz@colostate.edu.

colon cancer, and soft tissue sarcoma. These studies have been criticized because the statistical associations were weak, the results were imprecise, and causal criteria were not satisfied (6).

In terms of human exposure, contamination of ground water and soil from spills and negligence are of major concern because a small amount of ATRA can contaminate a rather large aquifer. Twenty pounds of ATRA evenly dispersed throughout an aquifer is sufficient to produce a concentration of 100 ng/mL in 2.6×10^6 gal of water (7). There is sufficient research to conclude that ATRA is a harmful toxicant in animal models (2,3), but the exact mechanisms of action and target tissues are still under investigation. Unlike other analytical methods that determine total ATRA equivalents using radiolabeled parent compound (8), this analytical method allows for quantitative analysis of ATRA and its chlorinated metabolites at levels of 100 ng/mL in plasma.

To date, diaminochlorotriazine (DACT), the major metabolite of ATRA found after a single oral gavage dose, has not been quantitatively identified in plasma. In urine, however, analytical methods for the determination of ATRA and its dealkylated metabolites have been developed using gas chromatography–mass selective detection (GC–MSD) with limits of quantitation (LOQ) for DACT equal to 1000 ng/mL (9). Previous studies have used enzyme-linked immunosorbent assays (ELISA) and high-performance liquid chromatography (HPLC) to detect ATRA and its chlorinated metabolites in urine and found no measurable concentrations of DACT (9). Detection of ATRA and its chlorinated metabolites in urine has been accomplished using GC–nitrogen-phosphorus detection (NPD), with an LOQ equal to 100 ng/mL (10).

By identifying ATRA and its individual metabolites in plasma, researchers will be able to model the kinetics of ATRA during in vivo and in vitro metabolism. This kinetic model could lead to a better understanding of ATRA's mechanism of action. By elucidating the exact mechanism of action of this compound, researchers can better understand its adverse endocrine effects. This analytical method is potentially applicable to other biological matrices (urine, liver, kidney, and brain) for quantitative identification of ATRA and its dealkylated metabolites at environmentally relevant concentrations.

Experimental

GC–MS conditions

A Hewlett-Packard (Palo Alto, CA) 5890 series II plus GC equipped with a model 5972 MSD in selected ion monitoring (SIM) mode were used to identify ATRA and *N*-dealkylated derivatized products. Identification and quantitation of these compounds was achieved by monitoring retention times and detection of characteristic target and qualifying ions in their respective mass spectra. The analytical column was a DB-17MS (30 m × 0.25-mm i.d., 0.25-mm film thickness, J&W Scientific, Folsom, CA) with a helium gas flow of 0.4 mL/min. Inlet and transfer line temperatures were set at 200°C and 300°C, respectively. The column oven was programmed to begin at 60°C, held for 1 min, then increased 15°C/min to 250°C and held for 4 min. The total run time was 17.67 min. Data was acquired in SIM mode with a dwell of 100 ms for each ion. Derivatized products were identified by monitoring retention times and SIM profiles for each analyte (Table I), with all compounds exhibiting excellent separation and resolution (Figures 2 and 3).

Standards and reagents

ATRA (97.1% purity) and DACT (96.8% purity) were gifts from Syngenta (Research Triangle Park, NC). Desethylatrazine (DE-ATRA, 99% purity), desisopropylatrazine (DI-ATRA, 99% purity), dimethyl sulfoxide (DMSO), redistilled diethyl ether (EE), acetone (Fisher Optima grade), *n*-hexane (Mallinckrodt nanograde), iodomethane (stabilized 99%), and tetrabutylammonium hydroxide (TBAOH) were all purchased from Fisher Chemical Company (Houston, TX). Cyanazine (CYA, 99% purity) was purchased from Sigma Chemical Co. (St. Louis, MO).

Table I. Retention Times and Target Ions of Methylated ATRA and Dealkylated Metabolites

Compound	Retention Time	Target Ion	Qualifier Ion #1	Qualifier Ion #2
Me-DACT	12.45 min	201	186	172
Me-DI-ATRA	12.66 min	200	215	186
Me-DE-ATRA	13.02 min	214	229	186
Me-ATRA	13.22 min	228	243	n/a
Me-CYA	15.63 min	253	268	240

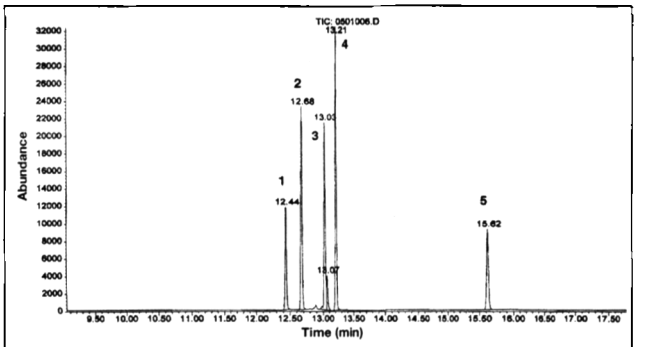

Figure 2. Derivatized 2500 ng/mL standard mix of Me-DACT (1), Me-DI-ATRA (2), Me-DE-ATRA (3), Me-ATRA (4), and Me-CYA (5).

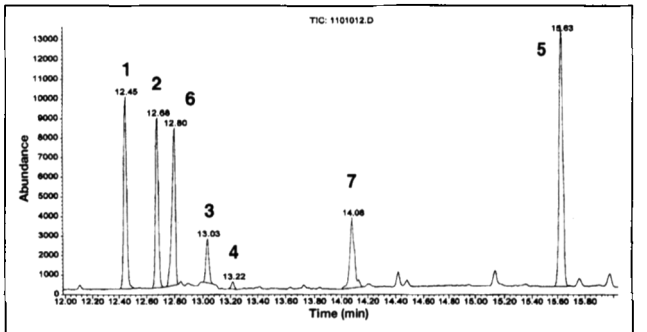

Figure 3. Derivatized (time-course) plasma sample showing detection of Me-DACT (1), Me-DI-ATRA (2), Me-DE-ATRA (3), Me-ATRA (4), Me-CYA (5), and two endogenous peaks associated with the sample matrix (6,7).

Standard preparation

Neat standards of ATRA, DE-ATRA, DI-ATRA, and CYA were prepared individually in acetone at 1.0 mg/mL and kept in glass vials with PTFE closure at 4°C. DACT was prepared in DMSO at 1.0 mg/mL. A mixture of all compounds (except CYA) was prepared in acetone at a concentration of 10 µg/mL. CYA was diluted in acetone to a working concentration of 10 µg/mL. Serial working dilutions were prepared from the standard mix in acetone at concentrations ranging from 10 to 5000 ng/mL. Two calibration curves (10–100 ng/mL and 500–5000 ng/mL) were prepared to cover the quantitation range of all analytes. CYA was used as a surrogate standard to monitor method performance during extraction and derivatization.

Method development

Control plasma was collected from rats never exposed to ATRA. Plasma samples (100 µL) were fortified with known amounts of ATRA, DE-ATRA, DI-ATRA, DACT, and CYA and were extracted using EE. Analytes were determined in sets, each set consisted of 1 control blank and 10 fortified plasma samples. One milliliter of saturated sodium sulfate (Na_2SO_4) was added to all plasma samples to reduce miscibility with EE extraction solvent. Two milliliters of EE was added to plasma samples, which were then vortex mixed and placed on a rotary mixer at room temperature for 10 min. Samples were then centrifuged for 2 min to separate the EE phase. The EE phase was transferred to a clean glass centrifuge tube, and the extraction was repeated twice more for a final EE extraction volume of 6.0 mL. The extract was evaporated to dryness under a steady stream of nitrogen. Two hundred microliters acetone (stored on 4Å molecular sieves) was added to the dried extracts (to azeotrope any remaining water that would interfere with the derivatization procedure), and the samples were again taken to dryness under nitrogen.

Derivatization

Calibration standards were prepared from 10-µg/mL stock solutions by adding appropriate volumes to clean 10-mL culture tubes with subsequent evaporation to dryness under a nitrogen stream. DMSO (100 µL) was used to reconstitute samples and standards. Five microliters of TBAOH, 500 µL of *n*-hexane, and 30 µL of methyl iodide (CH_3I) were added, and samples and standards were allowed to rotate at room temperature for 30 min. After the 30-min rotation, 1.0 mL of deionized water was added to all samples and standards, and the methylated analytes (Figure 4) were extracted twice using 2.0 mL of *n*-hexane with each extraction. The *n*-hexane was then evaporated under nitrogen to < 100 µL and reconstituted to 100 µL in *n*-hexane. Samples and standards were injected onto a Hewlett-Packard 5890 series II plus GC with 5972 MSD in SIM mode (GC–MS-SIM).

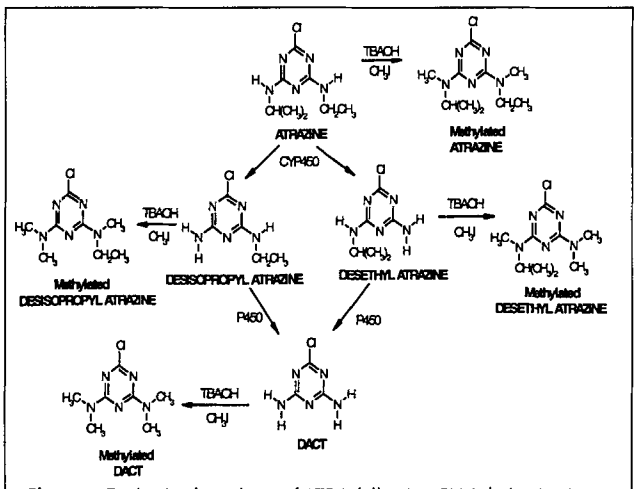

Figure 4. Derivatized products of ATRA following CH_3I derivatization.

Analytical method validation

A small-scale pilot study was performed in compliance with the Colorado State University Animal Care and Use Committee (ACUC) guidelines. Whole blood from uncannulated rats was obtained via arterial aortic puncture and was centrifuged immediately after collection. The plasma fractions were pooled and stored at –20°C until used as control plasma for quality control analysis. The described analytical method was employed to analyze plasma samples following a single oral gavage dose of 100 mg/kg of ATRA to cannulated female Sprague Dawley rats. Two groups of rats (group A equal to 3 dose and 1 control, group B equal to 3 dose and 1 control) were randomly assigned and dosed with either suspended ATRA in methylcellulose (MC) or MC only. All samples were centrifuged at 4°C at 8000 × *g* for 10 min to separate the plasma fraction within 8 h of collection over a 72-h time period. Time-course concentrations were determined using the described analytical method. This in vivo study validated the method efficacy in determining ATRA and dealkylated metabolite concentrations in plasma (Figure 3).

Method quantitation limit

Because of the relatively high atrazine doses administered in the time course study presented in this paper, quantitation limit determination was not a primary concern of this paper. For this study, a suitable quantitation limit was 100 ng/mL. Because this method used minimal cleanup (liquid–liquid extraction and no chromatographic cleanup), we anticipated some level of interference from matrix or reagents. To estimate interference levels in the current study, data were collected for control plasma samples. Plasma samples from the same pool were also fortified at 10, 100, and 2500 ng/mL to determine method performance.

Baseline interferences were determined with 25 control plasma samples. For DE-ATRA, DI-ATRA, and DACT, interferences were minimal, less than 5 ng/mL. For ATRA, interferences were considerably higher, approximately 10 ng/mL. In all cases, interfering peaks did not satisfy target/qualifier ion ratios for the respective analytes.

Forty 100-µL control plasma samples were fortified with ATRA, the three dealkylated metabolites, and the surrogate standard at 10 ng/mL, and all were extracted and analyzed using the described analytical method to determine the method quantitation limit (MQL). Baseline signal from control plasma interfered with quantitation by artificially enhancing target or qualifier ion abundance. This endogenous interference was easily recognized as it altered target/qualifier ion ratios. These

matrix interferences at times equated to at least half of the fortification level of 10 ng/mL and created problems in the quantitation of ATRA and the chlorinated metabolites. Matrix interferences often occur when working with biological matrices and vary from animal to animal. Because of variability in background matrix interference, the method was validated at 2500 and 100 ng/mL fortification of plasma samples (Figure 5 and Table II). Method performance at fortification levels of 2500 ng/mL and 100 ng/mL are shown in Table II. Average recoveries ranged from 84% to 97%, with percent coefficients of variation (%CV) of less than 20 for all compounds except ATRA (21%CV at 100 ng/mL). The higher %CV for ATRA is understandable based on interference in control samples. Method refinements are currently being investigated in our laboratory to lower the MQL, so this method can be used to identify environmentally relevant concentrations of ATRA and its dealkylated metabolites in biological samples. Matrix interferences did not play a significant role in quantitation of target compounds because of the high concentrations being analyzed in the experimental animal study performed in our laboratory.

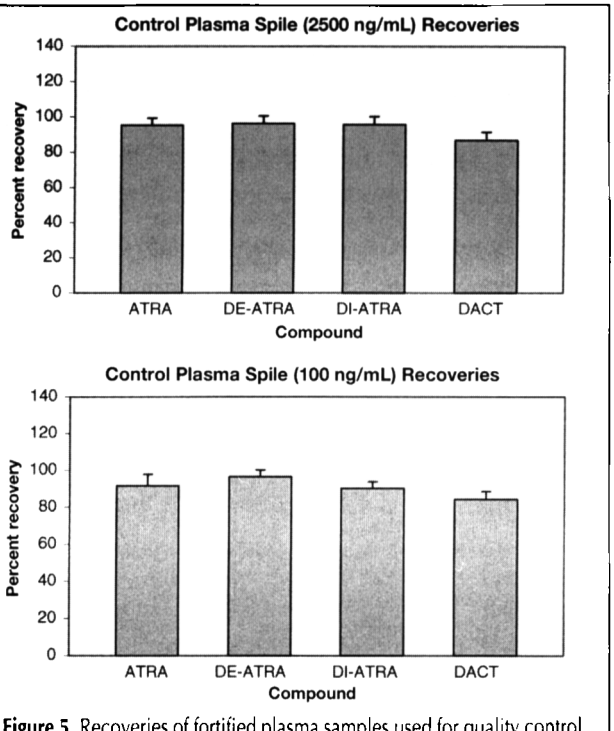

Figure 5. Recoveries of fortified plasma samples used for quality control in the small-scale pilot study.

Table II. Analyte Recoveries from Fortified Plasma

	ATRA	DE-ATRA	DI-ATRA	DACT
2500-ng/mL spike recoveries, *n* = 10				
Average	95	96	96	87
s	13	13	15	15
%CV	13	14	15	17
Std. Error	4.1	4.2	4.6	4.7
100-ng/mL spike recoveries, *n* = 13				
Average	92	97	90	84
s	19	11	12	13
%CV	21	12	13	16
Std. Error	6.1	3.6	3.7	4.3

Results and Discussion

From our initial small-scale pilot study, plasma levels of ATRA, DE-ATRA, DI-ATRA, and DACT were all detected and quantified using the described analytical method (Figure 6). Fortified plasma samples used for quality control purposes in the pilot study yielded recoveries ranging from 85% to 102% (Figure 5), and surrogate standard recoveries ranged from ± 20% of the fortified concentration indicating efficient extraction of the analytes of interest. When approaching the MQL of 100 ng/mL, matrix interferences must be closely investigated to ensure that the peak coeluting with the analyte of interest is not quantified as a false-positive result. Because matrix interferences vary within biological samples, ion ratios can be compared to known standard analyte ion ratios to ensure positive quantitation of the analytes of interest. Qualifier ion(s) can be deleted from the SIM profile if matrix interferences are greater than the MQL. Deletion of ions from the SIM reduces sensitivity and necessitates reestablishment of the MQL.

Conclusions

Unlike other methods that only determine total ATRA equivalents using radiolabeled parent compound or ELISA, this analytical method allows for individual quantitative analysis of parent compound and chlorinated metabolites to 100 ng/mL in plasma isolated from whole blood. Application of this analytical method to other biological matrices is currently under investigation in our laboratory. Identification and quantitation of triazine herbicides and individual chlorinated metabolites in plasma and other biological matrices could elucidate a better understanding of individual rates for chlorotriazine metabolite formation in plasma and aid in the extrapolation of an experimental internal dose to an environmental external exposure to ATRA.

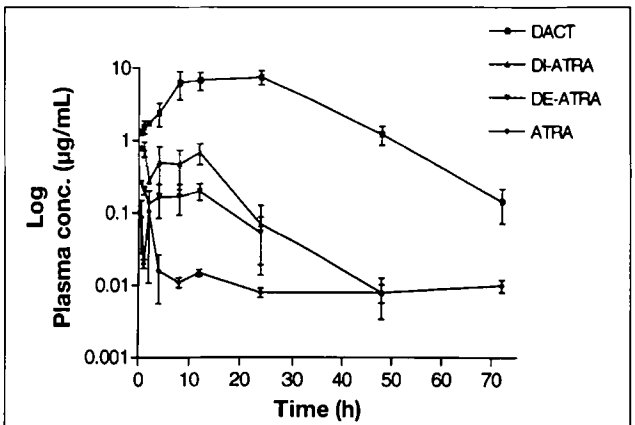

Figure 6. Plasma time-course data from a single oral gavage dose of 100 mg/kg.

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