Trace Level Analysis of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) and Its Biodegradation Intermediates in Liquid Media by Solid-Phase Extraction and High-Pressure Liquid Chromatography Analysis

Teresa M. Chow[1,*], Monte R. Wilcoxon[1], Marvin D. Piwoni[1], and **Neal R. Adrian[2]**

[1]The Illinois Waste Management Research and Information Center, Champaign, IL 61820 and [2]Engineer Research and Development Center, Army Corps of Engineers, Construction Engineering Research Laboratory, Champaign, IL 61826

Abstract

The use of solid-phase extraction for the analysis of liquid media containing low µg/L levels of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), mononitroso-RDX (MNX), dinitroso-RDX (DNX), and trinitroso-RDX (TNX) is examined. Aqueous samples (100 mL) consisting of water and a microbiological basal medium are spiked with known concentrations of RDX, MNX, DNX, and TNX. The compounds are extracted from the liquid media using a Porapak RDX cartridge and then eluted from the cartridge with 5 mL of acetonitrile. The eluent is concentrated to 1 mL before analysis by high-pressure liquid chromatography (HPLC). The method detection limits for RDX are 0.1 µg/L in water and 0.5 µg/L in the basal medium after a 100-fold concentration. For MNX, DNX, and TNX, the method detection limits are approximately 0.5 µg/L in water and approximately 1 µg/L in the basal medium after a 100-fold concentration. Interferences in the basal medium and a contaminant in the standard made quantitation for MNX and TNX, respectively, is less accurate below the 1 µg/L level. Solid-phase extraction of the liquid media gave good recoveries of nitramines and nitroso intermediates from a microbiological basal medium, allowing HPLC detection of RDX and the nitroso intermediates in the low µg/L (ppb) range.

Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a nitramine explosive, is used extensively by the military in shells, bombs, and demolition charges (1). Wastewater contaminated with RDX is generated during the production and demilitarization operations of conventional munitions (2). Improper disposal of this wastewater in the past has resulted in the contamination of soil and groundwater (3,4). Because RDX poses a risk to human health (5) and is potentially toxic to the indigenous species at contaminated sites (6), RDX is frequently removed from impacted areas.

The anaerobic biodegradation of explosives, including RDX, offers a potential solution for remediating explosives-contaminated sites and treating contaminated wastewater. The anaerobic biodegradation of RDX is well documented and has been reviewed (7). One biodegradation pathway involves the reduction of RDX to the nitroso derivatives prior to ring cleavage, but the other pathway results in the direct ring cleavage of the RDX ring (Figure 1). Adrian and Chow observed the three nitroso-RDX intermediates during the anaerobic biodegradation of RDX by a methanogenic mixed culture and identified hydroxylamino-dinitroso-1,3,5-triazine as a transient intermediate before the cleavage of the triazine ring (8). Two pure cultures were isolated from the mixed culture: a *Clostridium* species that degrades RDX when grown on yeast extract (9) and *Acetobacterium malicum* strain HAAP-1 that biodegrades RDX when grown in a mineral medium using hydrogen as the energy source (10). The *Clostridium* isolate produces significant amounts of mononitroso-RDX (MNX), dinitroso-RDX (DNX), and trinitroso-RDX (TNX), but only trace amounts of MNX are transiently formed during biodegradation of RDX by the *Acetobacterium* isolate.

The primary objective for this research was to develop a simple and relatively rapid method for the analysis of RDX and the nitroso-RDX intermediates in liquid media at low µg/L concentrations. This is useful for monitoring anaerobic biodegradation studies of RDX. We evaluated the use of solid-phase extraction

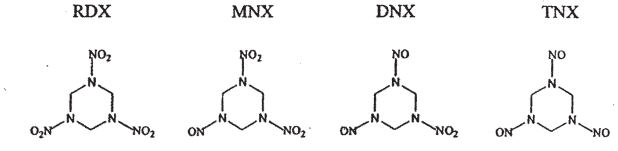

Figure 1. Structures of RDX and three nitroso-RDX intermediates.

*Author to whom correspondence should be addressed.

(SPE) for concentrating RDX from water and a microbiological basal medium before high-pressure liquid chromatography (HPLC) analysis. The method allowed for the detection of RDX and the nitroso-RDX intermediates at the low µg/L level when the analytes were concentrated 100-fold before analysis.

Experimental

Chemicals and reagents

Analytical standards for RDX were obtained from AccuStandard (New Haven, CT) and Supelco (Bellefonte, PA) and were greater than 99% pure. MNX, 98.4% pure, DNX, 67% pure and TNX, > 99.9% pure were obtained from SRI International (Menlo Park, CA). Basal medium was prepared and dispensed using strict anoxic techniques as previously described (11). All source chemicals were of the highest purity obtainable from Fisher Scientific (Pittsburgh, PA). HPLC-grade acetonitrile was obtained from Fisher Scientific (Pittsburgh, PA). HPLC-grade water was obtained from a Millipore (Bedford, MA) Ultrapure Water system (Milli-Q) in the laboratory. Waters Porapak RDX SPE cartridges (6 mL, 500 mg) were purchased from Waters.

SPE

Water and basal media were spiked with RDX to final concentrations of 0.1, 0.5, 1.0, 5.0, and 10.0 µg/L by diluting a 1000 µg/L RDX stock 100-fold and then making serial dilutions with it using volumetric flasks. A mixture of MNX (88 µg/L), DNX (88 µg/L), and TNX (103 µg/L) was used to spike water and basal media to final concentrations of approximately 0.5, 1, 5, and 10 µg/L (Table I) by diluting 200×, 100×, 20×, and 10×, respectively, into 100-mL volumetric flasks with water or basal media. Porapak cartridges were conditioned by passing 15 mL of acetonitrile followed by 10 mL of methanol through at gravity flow rate, then rinsing with 30 mL of deionized water at a flow rate of approximately 10 mL/min. One hundred milliliters of the liquid media were passed through the cartridges at flow rates of approximately 4.5 mL/min using a Supelco Visiprep 12-port SPE manifold attached to the house vacuum. After all the samples were extracted, the cartridges were rinsed with 15 mL of water and air was drawn through for 2 min to dry the cartridges. The extracted analytes were then eluted off the cartridges using 5-mL portions of acetonitrile by gravity flow into glass vials. Silicone tubing attached to a cylinder of compressed nitrogen (ultrahigh-purity grade) and fitted with glass pipets was used to concentrate the collected eluents to approximately 0.5 mL. They were then transferred to 1 mL volumetric flasks by glass pipets and brought up to volume using acetonitrile before HPLC analysis.

Table I. Percent Recoveries of MNX, DNX, and TNX Extracted from Water and Basal Medium

	(A) Concentrations in water (MNX, DNX, TNX µg/L)			
Analytes	(0.44, 0.43, 0.51)	(0.88, 0.86, 1.0)	(4.4, 4.3, 5.1)	(8.8, 8.6, 10.3)
MNX	105%	88%	97%	101%
DNX	116	95	117	120
TNX	43	52	99	102
	(B) Concentrations in basal medium (MNX, DNX, TNX µg/L)			
Analytes	(0.88, 0.86, 1.0)	(4.4, 4.3, 5.1)	(8.8, 8.6, 10.3)	
MNX	120%	102%	125%	
DNX	123	109	116	

Chromatography

HPLC analysis was performed on a Waters Alliance 2695 Separation Module with autosampler and a 996 photodiode array detector using a Waters Symmetry C8 reversed-phase column (3.9 × 150 mm, 5 µm) with a C8 Sentry guard column. The following conditions were used: mobile phase, methanol–water (40:60); injection volume, 25 µL; and flow rate, 1 mL/min. Photodiode data were collected from 210 to 300 nm. The chromatograms were extracted at 235 nm for RDX and at 250 nm for the nitroso derivatives. Identification of the extracted RDX, MNX, DNX, and TNX was carried out by comparing retention times and spectra from 210 to 300 nm to those of authentic standards before extraction.

Results and Discussion

Figure 2A shows the HPLC chromatogram of a 40 µg/L RDX standard. Based upon the analyses of an ultrapure water blank and the low level standards, the instrument detection limit for

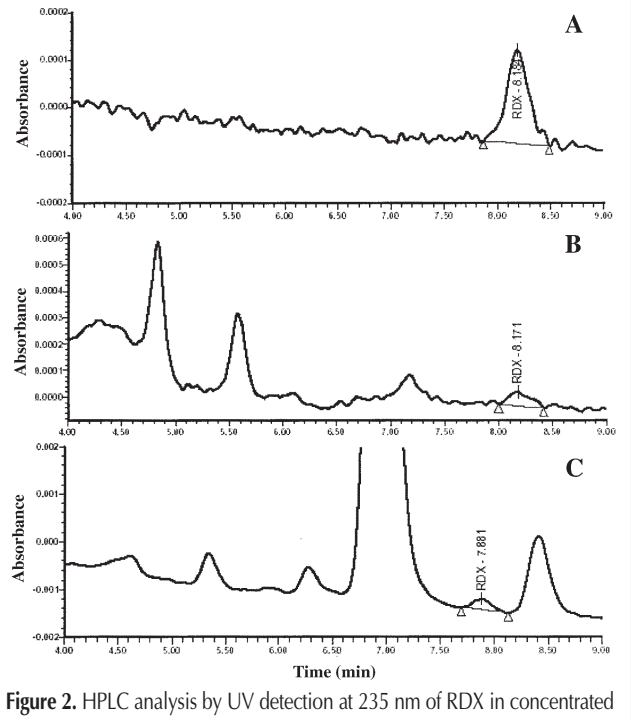

Figure 2. HPLC analysis by UV detection at 235 nm of RDX in concentrated eluent obtained from SPE of liquid media. (A) 40 µg/L of RDX analytical standard, (B) eluent from extraction of 100 mL 0.1 µg/L of RDX in water, and (C) eluent from extraction of 100 mL 0.5 µg/L of RDX in basal salt medium.

RDX, defined as three times the standard deviation of the noise divided by the slope of the calibration curve, was 11 µg/L with our HPLC conditions and a 25-µL injection volume. Recoveries of RDX extracted from water for all 100 mL samples were as follows: 93% for 0.1 µg/L, 90% for 0.5 µg/L, 86% for 1.0 µg/L, 94% for 5.0 µg/L, and 93% for 10.0 µg/L of RDX in the water sample. Quantitation was calculated using a five-point calibration curve. Concentrating the 100-mL water sample spiked with 0.1 µg/L to 1 mL gave a final theoretical RDX concentration of 10 µg/L in the sample extract, at the detection limit for RDX. The extract concentration was measured at 9.3 µg/L giving a 93% recovery (Figure 2B). We did observe some interferences from the extracted basal medium blank. They were attributed to the vitamins that are included in the medium necessary for the growth of bacteria. The recoveries of RDX extracted from basal medium were generally lower. They were: 75% for 0.5 µg/L, 78% for 1.0 µg/L, 88% for 5.0 µg/L, and 87% for 10 µg/L. The retention time for RDX in the extract from basal medium decreased to 7.88 min from 8.18 min in water. The peak was confirmed to be RDX by its spectrum. The shift was probably attributable to other components in the extract from the basal medium (Figure 2C). The lowest level at which RDX could be detected in the basal medium after extraction was 0.5 µg/L, at 75% recovery.

A concentrated mix standard (880 µg/L MNX, 860 µg/L DNX and 1030 µg/L TNX) was prepared by mixing the three nitroso analytes in acetonitrile, which was then diluted 2×, 10×, and 20× for the calibration curve. Figure 3A shows the HPLC chromatogram of the mix standard (440 µg/L MNX, 430 µg/L DNX, and 515 µg/L TNX). There were a few extra peaks in the standard because these compounds were not 100% pure. One contaminant came from the MNX standard and interfered with TNX quantitation in the mixed standard. Based on the analyses of an ultrapure water blank and the low level standards, the instrument detection limits for MNX, DNX, and TNX were 65, 66, and 30 µg/L, respectively. Recoveries of these nitroso-RDX intermediates extracted from water for all 100 mL samples processed are listed in Table IA for all spiked levels quantitated against a three-point calibration curve. MNX, DNX, and TNX were all detected in the sample spiked with approximately 0.5 µg/L of each compound, the lowest concentration that was extracted. Figure 3B shows the extracted analytes from water spiked at 0.88, 0.86, and 103 µg/L concentration. The SPE step seems to remove some of the contaminating peaks observed in the mixed standard, resulting in a cleaner chromatogram except that the one contaminant interfering with TNX in the mixed standard was still present. However, this peak was smaller, therefore integration at low concentrations was more accurate. The interferences extracted from the basal medium made integration problematic for MNX at or below 0.88 µg/L. The method detection limits for these 3 analytes were about 1 µg/L. The recoveries of MNX, DNX, and TNX extracted from basal medium, based on a three-point calibration curve, are listed in Table IB. The nitroso peaks were confirmed by comparing their spectra with those of the standards. Figure 3C shows the chromatogram of the three nitroso compounds at approximately 5-µg/L concentrations extracted from 100 mL of basal solution.

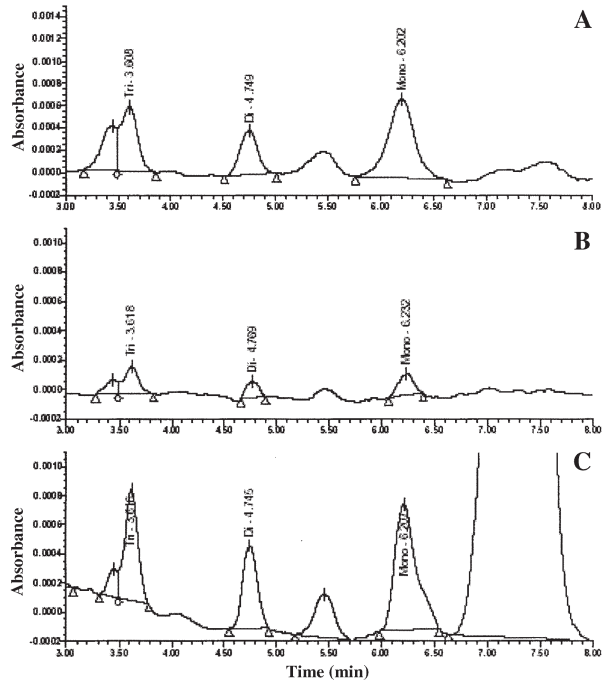

Figure 3. HPLC analysis by UV detection at 250 nm of nitroso-RDX intermediates in concentrated eluant obtained from SPE of liquid media. (A) 440, 430, 515 µg/L MNX, DNX, TNX standard; (B) eluent from extraction of 100 mL 0.88, 0.86, and 1.0 µg/L of MNX, DNX, and TNX in water; (C) eluent from extraction of 100 mL of 4.4, 4.3, and 5.1 µg/L of MNX, DNX, and TNX in basal medium.

Conclusion

We evaluated SPE as a preconcentration step for analyzing RDX, MNX, DNX, and TNX at low µg/L levels in water and a bacterial basal growth media. The method detection limit for RDX was 0.1 µg/L in water but was higher at 0.5 µg/L in the basal medium after a 100-fold concentration of the analytes. MNX, DNX, and TNX provide a weaker response than RDX, with method detection limits close to 0.5 µg/L in water and approximately 1 µg/L in basal medium after a 100-fold concentration. The interferences in the basal medium and a contaminant in the standard made quantitation for MNX and TNX, respectively, less accurate below the 1-µg/L level, although future work optimizing the chromatographic conditions could possibly improve separation, making quantitation more accurate at low µg/L levels. In general, the Porapak RDX Sep-Pak Vac cartridges, specifically designed for preconcentrating nitroaromatics and nitramines in water (12), gave good recoveries of these explosives at µg/L (ppb) concentrations from a microbiological basal medium, a much more complex matrix than water.

References

1. B.T. Fedoroff and O.E. Sheffield. Cyclotrimethylenetrinitramine, cyclonite or RDX. In *Encyclopedia of Explosives and Related Items.* Vol. 3. Picatinny Arsenal, Dover, NJ, 1966.
2. M. Qazi, B. Froward, M. Schor, and B. Nelson. *Pink water treatment options.* Final report ADA295802. National Technical Information

Service. Springfield, VA, 1995, pp. 1–99.
3. R. Haas, E.V. Löw Schreiber, and G. Stork. Conception for the investigation of contaminated munitions plants. 2. Investigation of former RDX-plants and filling stations. *Fresenius' J. Anal. Chem.* **338:** 41–45 (1990).
4. C.A. Myler and W. Sisk. "Bioremediation of explosives contaminated soils (scientific questions/engineering realities)". In *Environmental Bio/Technology for Waste Treatment*. G.S. Sayler, R. Fox, and J.W. Blackburn, Eds. Plenum Press, New York, NY, 1991, pp. 137–46.
5. C. Smith-Simon and S. Goldhaber. *Toxicological profile for RDX*. Agency for Toxic Substances and Disease Registry, U.S. Department of Health and Human Services, Atlanta, GA, 1995.
6. S.S. Talmage, D.M. Opresko, C.J. Maxwell, C.J. Welsh, F.M. Cretella, P.H. Reno, and R.B. Daniel. Nitroaromatic munition compounds: environmental effects and screening values. In *Rev Environ. Contam. Toxicol,* Vol. 161. G.W. Ware, Ed. Springer, New York, NY, 1999.
7. J. Hawari, S. Beaudet, A. Halasz, S. Thiboutot, and G. Ampleman. Microbial degradation of explosives: biotransformation versus mineralization. *Appl. Micribiol. Biotechnol.* **54:** 605–18 (2000).
8. N. R. Adrian and T. Chow. Identification of hydroxylamino-dinitroso-1,3,5-triazine as a transient intermediate formed during the anaerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5- triazine. *Environ. Tox. & Chem.* **20:** 1874–77 (2001).
9. C.M. Arnett and N.R. Adrian. Isolation and characterization of a *Clostridium* species that degrades hexahydro-1,3,5-trinitro-1,3,5-triazine, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, and 2,4,6-trinitrotoluene. Abstacts of the Annual Meeting of the American Society for Microbiology: Q-274, American Society for Microbiology, Orlando, FL, 2001, 639.
10. N.R. Adrian and C.M. Arnett. Anaerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by *Acetobacterium malicum* strain HAAP-1 isolated from a methanogenic mixed culture. *Current Microbiology* **48:** 332–40 (2004).
11. N.R. Adrian and C.M. Arnett, and R.F. Hickey. Stimulating the anaerobic biodegradation of explosives by the addition of hydrogen or electron donors that produce hydrogen. *Water Research* **37:** 3499–3507 (2003).
12. Waters. Improved "Analysis and Identification of Nitramine and Nitroaromatic Explosives". *Waters Column* Special ed. Waters, Milford, MA, 1994, pp. 12–14.

Manuscript accepted September 3, 2004.