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Short Communication

Separation and quantitation of nitrobenzenes and their reduction products nitroanilines and phenylenediamines by reversed-phase high-performance liquid chromatography

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method for the separation and quantitation of a mixture consisting of nitrobenzene, dinitrobenzene isomers, 1,3,5-trinitrobenzene and their reduction products: aniline, nitroanilines and phenylenediamines has been developed. The method is sensitive and highly reproducible. The mixture is resolved on a Zorbax C_8 column with 0.1% triethylamine and methanol as the mobile phase. The detection limits for individual chemicals at 254 nm are in the range of 25–50 ng.

INTRODUCTION

A number of nitroaromatic compounds have been shown to form as by-products during the manufacture of the explosive 2,4,6-trinitrotoluene (TNT) [1–3]. Among these by-products, dinitrobenzene isomers (*o*-, *m*-, *p*-DNB), 1,3,5-trinitrobenzene (TNB) and 3,5-dinitroaniline (*m*-DNAN) are frequently detected in waste water discharged from TNT production facilities and

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also at open-burn and open-detonation grounds and neighboring areas [1–3]. In addition, the formation of TNB and DNB in the environment by photochemical conversion of discharged TNT and 2,4-dinitrotoluene (2,4-DNT) has also been reported [3]. TNB, DNAN and DNB are mutagenic to *Salmonella typhimurium* [4,5] and were shown to be toxic to various animal species and microorganisms [3]. The DNB isomers are potential inducers of methemoglobinemia in experimental animals and in humans [6–10]. One isomer, *m*-DNB, causes splenomegaly and testicular atrophy in rats [11,12]. Several DNB isomers and TNB form covalent adducts with rat blood proteins [13,14] and with soft tissue DNA [14]. Reduction of the nitro group of DNB isomers and TNB to the corresponding nitroanilines by isolated hepatocytes [15] and by cell free extracts from *Veillonella alkalescence*, *Nocardia* species or phenol adapted bacteria from sewage sludge has been reported [16–18].

In recent years, several liquid chromatographic or gas chromatographic, mass spectrometric methods have been reported for the separation and quantitation of nitroaromatic compounds and their reduction products either obtained commercially or extracted from contaminated soil/water [19–24]. Several metabolites of nitrobenzene (NB) [25] and the DNB isomers [15] were resolved on reversed-phase high-performance liquid chromatography (HPLC). However, a single chromatographic method for the resolution of a mixture containing NB, DNB isomers, TNB, and the reduction products such as nitroanilines and phenylenediamines (*o*-, *m*-, *p*-isomers), is currently not available. In this communication we describe a reversed-phase HPLC method suitable for the resolution and quantitation of such mixtures.

EXPERIMENTAL

Chemicals

NB, *o*-DNB, *m*-DNB, *p*-DNB, aniline (AN), 2-nitroaniline (*o*-NAN), 3-nitroaniline (*m*-NAN), 4-nitroaniline (*p*-NAN), 1,2-phenylenediamine (*o*-PD), 1,3-phenylenediamine (*m*-PD), 1,4-phenylenediamine (*p*-PD), *m*-DNAN and 4-nitroacetanilide (4-NACAL) were obtained (97–99% purity) from Aldrich (Milwaukee, WI, USA). TNB was supplied 99% pure by the US Army Biomedical Research and Development Laboratory, Fort Detrick, Frederick, MD, USA. Methanol (HPLC grade) was obtained from Burdick and Jackson (Muskegon, MI, USA) and triethylamine (sequanal grade) was supplied by Pierce (Rockford, IL, USA) Glass-distilled water was prepared in our laboratory.

HPLC standards

Stock solutions of the individual chemicals were prepared in methanol (100 μg/ml). All stock solutions except *p*-PD (prepared as needed) were stable for at least 2 weeks at −20°C. A working standard mixture (0.5 μg/ml) was prepared daily by mixing 50 μl from each stock solution into a separate vial which was brought up to a final volume of 10 ml.

High-performance liquid chromatography

Millipore/Waters (Milford, MA, USA) HPLC equipped with a 600E solvent controller, a 484 tunable absorbance detector, a Berthold HPLC radioactivity monitor (LB 506 C-1) and interfaced with an Epson Equity III computer was used. Integration and peak quantitation were achieved by using Berthold HPLC Program version 1.43. The effluent was monitored at 254 nm (0.1 AU full scale) and separations were achieved on a Zorbax C_8 column (5 μm particle size, 25 cm × 9.4 mm) connected behind a cartridge guard column (1.25 cm × 4 mm).

Mobile phase and gradient conditions: gradient I. Solvent A: 0.1% triethylamine in water (pH 6.5–6.8); solvent B: methanol. Flow-rate was 3 ml/min and the gradient curve was set at G-5. A–B (90:10) was used as initial condition. The methanol concentration was increased from 10 to 30% B in 10 min, then to 50% B in 50 min and 50% B to 100% B in 5 min. After 5 min at 100% B, the gradient was reversed to initial conditions in 10 min and equilibrated for an additional 10 min before the next sample was injected. Solvents A and B were degassed every day by filtering through 0.22-μm membrane filters. During operation the solvents were sparged with helium gas (60 ml/min).

Mobile phase and gradient conditions: gradient II. This gradient was developed for the separation and quantitation of 4-NACAL from a mixture. A–B (80:20) was used as initial condition. The methanol concentration was linearly (G-6) increased from 20% to 50% in 15 min then to 65% in 25 min, and finally to 100% in 10 min. The column was washed for an additional 5 min and brought back to initial conditions (20% B) in 10 min by reverse gradient and was equilibrated for an additional 10 min at initial conditions before the next sample was injected. The flow-rate was 3 ml/min.

RESULTS AND DISCUSSION

In our early attempts with methanol–water gradients as a mobile phase, phenylenediamines were eluted as broad peaks with significant trailing and with elevated base line. However, the introduction of 0.1% triethylamine (pH 6.5–6.8) in water (solvent A) dramatically improved both the resolution (baseline separation) and the sharpness of all three phenylenediamine peaks. Further increasing the triethylamine concentration to 0.2% did not result in any improvement in the peak resolution or sharpness. Hence, the triethylamine concentration in water was maintained at 0.1% throughout (solvent A). For the separation and quantitation of nitrobenzenes and their possible reduction products on HPLC, several methanol–0.1% triethylamine gradients were employed. Two of the gradients that separated the mixture most effectively (gradients I and II) are described. A mixture, consisting of 13 components (nitrobenzenes, nitroanilines and phenylenediamines), was resolved using gradient I and is shown in Fig. 1. 4-NACAL, a potential metabolite of 1,3-DNB [26] did not resolve from TNB on gradient I. Therefore, gradient II was developed to resolve 4-NACAL from the mixture and is shown in Fig. 2. In gradient II, *o*-NAN co-eluted with *p*-DNB and *o*-DNB co-eluted with *m*-DNB. Therefore, *o*-NAN and *o*-DNB were not included in the mixture. Individual peak identification and their retention times for figures one and two are presented in Table I.

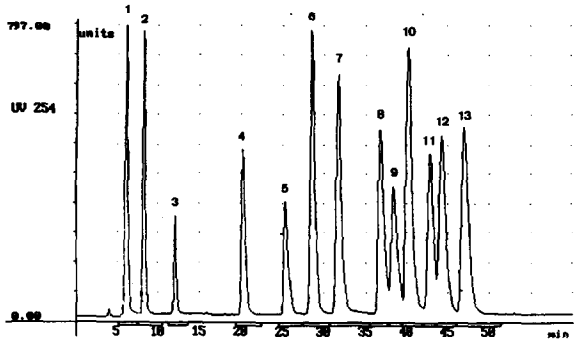

Fig. 1. Separation of a mixture containing nitrobenzenes and their reduction products on Zorbax C_8 column (25 cm × 9.4 mm). Injection volume: 100 μl, each peak represents 5 μg. For other details see Experimental section.

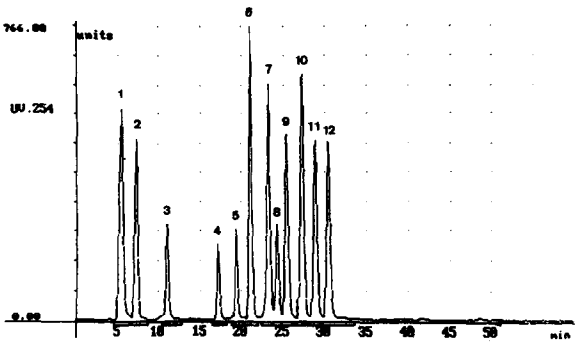

Fig. 2. Separation of a mixture containing nitrobenzenes and reduction products on Zorbax C_8 column (25 cm × 9.5 mm). Injection volume: 100 μl, each peak represents 5 μg. For other details see Experimental section.

Linear regression analysis. Regression analysis was carried out on each peak area against concentration and the correlation co-efficient was between 0.997 and 0.999 over the concentration range of 25–500 ng.

CONCLUSIONS

The method described here allowed a complete resolution and rapid determination of nitrobenzenes and their reduction products and the detection limit was 25–50 ng per chemical. The standard deviation (±S.D.) values presented from six different HPLC runs suggest that the retention times of individual peaks in Figs. 1 and 2 are highly reproducible. This method may be

TABLE I

PEAK IDENTIFICATION AND THE RETENTION TIMES OF CHROMATOGRAMS FROM FIGS. 1 AND 2

Gradients 1 and 2

Peak No.	Compound	Retention times (min:s)[a]	
		Gradient 1	Gradient 2
1	*p*-PD	6:42 ± 0:20	5:25 ± 0:11
2	*m*-PD	9:00 ± 0:24	7:15 ± 0:10
3	*o*-PD	12:54 ± 0:24	11:05 ± 0:05
4	AN	21:00 ± 0:18	17:00 ± 0:15
5	*p*-NAN	26:00 ± 0:18	19:18 ± 0:12
6	*m*-NAN	29:00 ± 0:18	21:06 ± 0:24
7	TNB	32:12 ± 0:18	23:12 ± 0:18
8	*p*-DNB/4-NACAL[b]	37:12 ± 0:12	24:30 ± 0:09
9	*o*-NAN/*p*-DNB[b]	38:37 ± 0:12	25:30 ± 0:19
10	*m*-DNB	40:27 ± 0:12	27:14 ± 0:12
11	*o*-DNB/*m*-DNAN[b]	43:00 ± 0:12	28:46 ± 0:07
12	*m*-DNAN/NB[b]	44:17 ± 0:15	30:22 ± 0:09
13	*NB*	47:06 ± 0:12	

[a] Mean ± S.D. from six HPLC runs.
[b] For gradient 2 (Fig. 2) only.

used to screen and determine the environmental fate of nitrobenzenes that were generated during munition manufacture and discharged into surface waters and soils. It can also be employed for the isolation, identification and quantitation of mammalian and plant metabolites of nitrobenzenes.

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DISCLAIMER

This document has been reviewed in accordance with US Environmental Protection Agency (EPA) and US Army policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use. The findings in this report are not to be construed as official US EPA/US Army position unless so designated by other authorized documents.

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