Journal of Chromatography, 133 (1977) 59–66

CHROM. 9700

A NITROSAMIDE-SPECIFIC DETECTOR FOR USE WITH HIGH-PRESSURE LIQUID CHROMATOGRAPHY*

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(First received May 10th, 1976; revised manuscript received August 13th, 1976)

SUMMARY

A highly specific automated method for identification of nitrosamides has been developed for use in conjunction with reversed-phase high-pressure liquid chromatography. The method is based on the well-known Griess reagent, and is capable of sensitivities of 0.5 nmoles of injected nitrosamide in most cases. The method may be used for analysis of nitrite, nitrosamides, nitrosocarbamates, and alkylnitrites.

INTRODUCTION

Most N-nitroso compounds can be readily analyzed by gas chromatography (GC). Most N-nitrosoamides, on the other hand, are not generally amenable to direct analysis by GC, either because of their low volatility or thermal instability, or both. These compounds, *i.e.*, N-nitrosoureas, -carbamates, -guanidines, *etc.*, are of potential environmental importance since many of their unnitrosated precursors are widely used as herbicides or pesticides and could conceivably enter the human food chain. High-pressure liquid chromatography (HPLC), operating at or near room temperature, affords the analyst a powerful technique for the analysis of this type of compound. Indeed, in 1975 Heyns and Röper[1] reported the use of reversed-phase HPLC for analysis of N-nitrosoureas and -urethanes.

We describe here a novel detector for HPLC which is completely specific for compounds which hydrolyze in dilute acid to give nitrite. The nitroso-compound detector (NOD) is based on the well-known Griess reagent as described by Preussmann and Schaper-Drukrey[2] for specific analysis of nitrosamides. Automation of the Griess reagent method to determine nitrite in soil and water has been reported[3]. The nitrosamide is cleaved in dilute acid at 90°. The nitrite released (as HONO) diazotized

* Research supported jointly by the Energy Research and Development Administration and the National Cancer Institute under contract with Union Carbide Corporation.

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sulfanilic acid, which then couples with naphthylethylenediamine to give a purple colored dye. Daiber and Preussmann's method[4] for analysis of N-nitroso compounds with the Griess reagent after ultraviolet (UV) irradiation was modified and automated by Fan and Tannenbaum[5]. This procedure is general for all N-nitroso compounds and requires a relatively lengthy irradiation time (30 min) for each sample.

We required a very specific, rapid automated analysis to identify individual nitrosamides and other easily denitrosated compounds in mixtures as they are eluted in HPLC.

We have automated and adapted the Griess reagent procedure so that the flow system accepts the effluent directly from the chromatograph. The NOD is specific for all compounds which are cleaved by dilute hydrochloric acid to give nitrite. This includes nitrosamides, nitrosoureas, nitrosocarbamates, *etc.*, and has also proven useful for the analysis of alkyl nitrites and inorganic nitrite itself in a reaction mixture. Simple aliphatic nitrosamines are not detected. The analysis is rapid (3 min residence time) and highly reproducible.

EXPERIMENTAL

Reagents and chemicals

Sulfanilic acid, obtained from Aldrich, was recrystallized from hot water before use. A solution (0.5%) in 30% acetic acid, was prepared by stirring for 15 min and filtering. N-1-naphthylethylene diamine dihydrochloride (Eastman-Kodak) was used without further purification as a 0.1% solution in 30% acetic acid.

Nitrosamides and alkyl nitrites tested were prepared in this laboratory by conventional procedures. Precursors were obtained from commercial sources.

Equipment

A Waters Assoc. Model 202 high-pressure liquid chromatograph equipped with a Model 660 solvent programmer and a UK6 loop injector was used. The column used throughout this work was 30 cm × 4 mm μBondapak C_{18} (Waters Assoc.) with either acetonitrile–water or *n*-propanol–water as eluent. Solvents were reagent grade, and were filtered through 0.5-μ type FH Millipore filters before use, but were not purified further.

Automated analysis

A Technicon AutoAnalyzer Pump I was used in conjunction with a Schoeffel SF 770 Spectroflow monitor. The flow system is diagrammed in Fig. 1.

The HPLC eluent was segmented by an air bubble and reagents were introduced by the pump; the mixture passed into a mixing coil (7 turn, 1.6 mm I.D.) and then into a 90° heating bath (1.6 mm × 10 ft. coil). The solution was then cooled by passage through a 3-ft. PTFE capillary (0.063 in. O.D., 0.026 in. I.D.). It was then led through a debubbler and into the SF 770, which was monitoring at 550 nm. Total residence time in the flow system was 3 min, *i.e.*, the time elapsed from the appearance of a given peak on the HPLC detector (254 nm) to the appearance of the corresponding peak on the monitor at 550 nm.

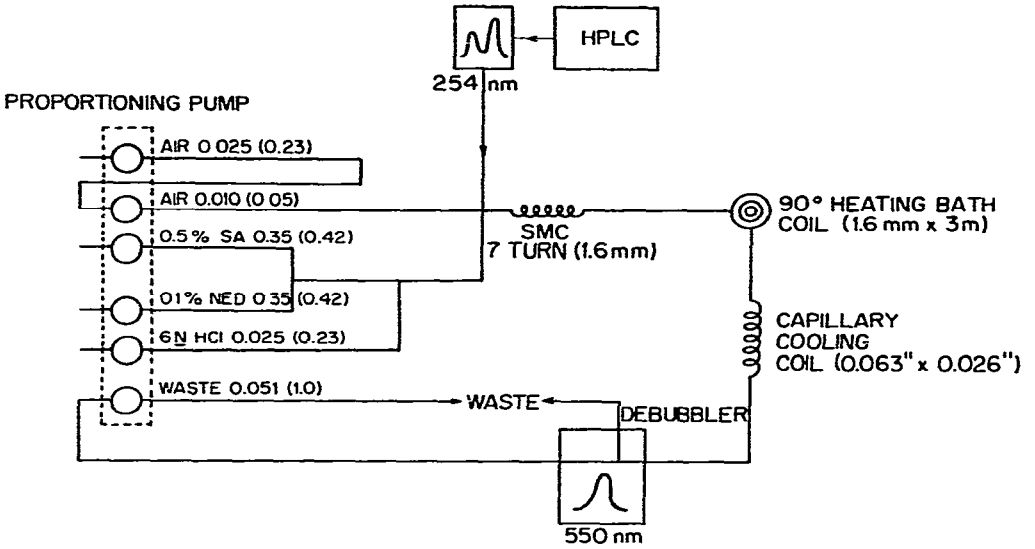

Fig. 1. The NOD flow system. The sample is injected into the HPLC; separation occurs on the column and is recorded by a 254 nm detector. The components then pass into the NOD where only nitrosamides are detected. SA = sulfanilic acid; NED = naphthylethylene diamine.

RESULTS AND DISCUSSION

In developing the procedure, both the choice of appropriate HPLC conditions and the chemistry of a useful automated flow system had to be considered. Since the NOD chemistry is aqueous, the use of a reversed-phase LC column was essential. The μBondapak C_{18} column with acetonitrile–water mixtures provided relatively rapid elution of various nitrosamides with good resolution (no retention times greater than 15 min), but two difficulties were encountered in the detector flow system. First, acetonitrile interferes with the color reaction, 10% acetonitrile in water reducing the absorbance by about 50%. Second, since the boiling point of acetonitrile (81°) is below the ideal temperature of the heating bath (90°), the acetonitrile tended to vaporize in the heating bath, and precipitously forced the contents of the heating coil to flash out, and, thus, inhibited color formation. We found *n*-propanol to be a suitable alternate solvent, percentages ranging from 5% to 80% having no effect on color formation. Its higher boiling point (90°) alleviated the heating bath problems, but in some respects it is a less desirable LC solvent than acetonitrile. While propanol–water mixtures were quite effective in achieving separations, the high UV density of propanol led to extreme baseline rise off-scale when programming was attempted. That this baseline rise did not affect the flow system or reflect elution of built-up column residue is demonstrated in Fig. 2.

The injected solution contained N-nitrosomethomyl, N-nitrosocarbaryl, and N-nitrosolandrin. Solvent programming from 25% to 70% *n*-propanol over 15 min, using a concave gradient (see Fig. 2), gave an excellent separation of the three compounds as shown by the NOD trace, while the UV detector baseline went off-scale before the elution of the N-nitrosocarbaryl and the N-nitrosolandrin.

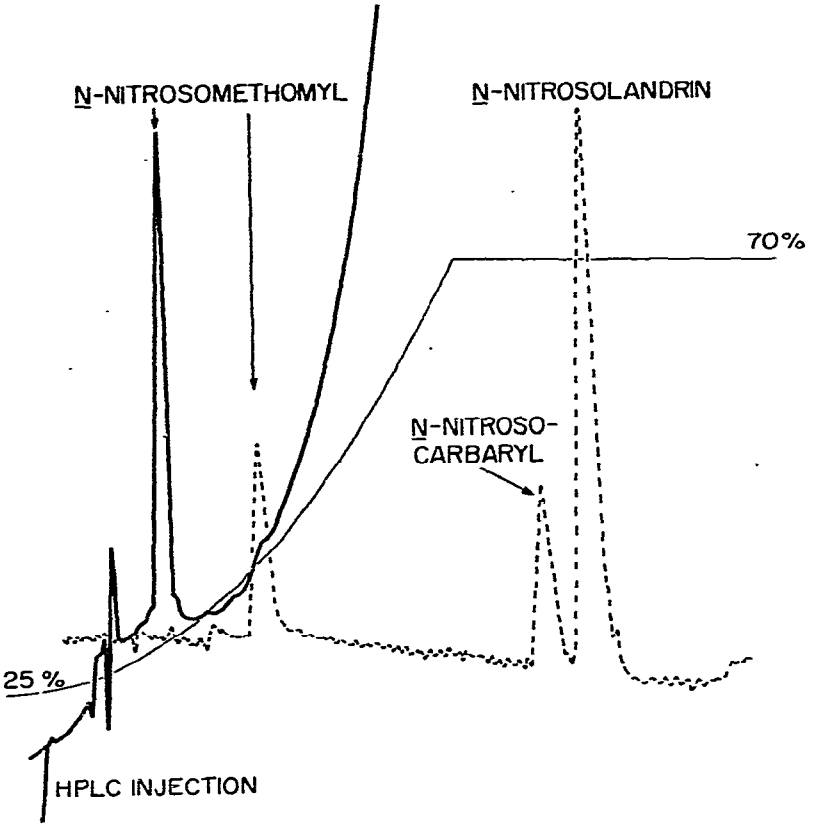

Fig. 2. Comparison of HPLC and NOD with solvent programming using propanol–water mixtures. The HPLC baseline goes off-scale, but the colorimeter baseline is only slightly affected. ———, HPLC trace; ———, HPLC solvent program; — — —, colorimeter trace.

A variety of nitrosamides, most of which had been synthesized for biological testing, were used to demonstrate the general applicability of the NOD. Sensitivities vary, due to the relative labilities of the N–NO bonds in the tested compounds.

A summary of compounds tested is given in Table I. Each data point is the average of at least two injections. The data were treated by a least squares analysis and correlation coefficients ranged from 0.96 to 0.999, indicating excellent linearity over the concentration range examined, *viz.*, 0.5–20 nmoles or 50–2000 ng injected. (For nitrosocaprolactam, the range was 1.8–64.5 nmoles with a correlation coefficient of 0.998.) The degree of linearity is illustrated graphically in Fig. 3.

The relative efficiency of the detector procedure for five representative compounds (nitrosomethylurea, nitrosoethylurea, N-ethyl-N′-nitro-N-nitrosoguanidine, nitrosomethylurethane, trimethylnitrosourea) was determined by comparing peak areas with those resulting from injection of a standard solution of sodium nitrite (prepared by dissolving a weighed amount of silver nitrite in hot water, diluting the solution, precipitating silver chloride by the addition of sodium chloride, and clarifying before injection)[6]. The efficiencies ranged from 87 to 108%.

TABLE I

SENSITIVITY OF NOD IN TERMS OF NANOGRAMS OF NITROSO COMPOUNDS INJECTED INTO THE HPLC

Sodium nitrite was either Fisher Reagent Grade or generated from silver nitrite synthesized in this laboratory, since silver nitrite is a more suitable analytical standard. The various nitrosamides tested were synthesized in this laboratory and their purity, assayed by UV spectroscopy and thin-layer chromatography, was satisfactory by the usual criteria.

Compound	*Mol. wt.*	*Maximum sensitivity*	
		ng injected	*nmoles*
Sodium nitrite	69	35	0.5
N-Nitroso-N-methylurea	103	50	0.5
N-Nitroso-N-butylurea	131	304	2.3
N-Ethyl-N'-nitro-N-nitrosoguanidine	161	126	0.78
N-Nitroso-N-methylurethane	132	150	1.1
N-Nitroso-N-ethylurea	117	125	1.1
N-Nitrosopyrrolidone	114	175	1.5
N-Nitrosocaprolactam	152	280	1.8
N-Nitrosomethomyl	191	300	1.6
N-Nitrosolandrin	222	1000	4.5
N-Nitrosocarbaryl	230	350	1.5

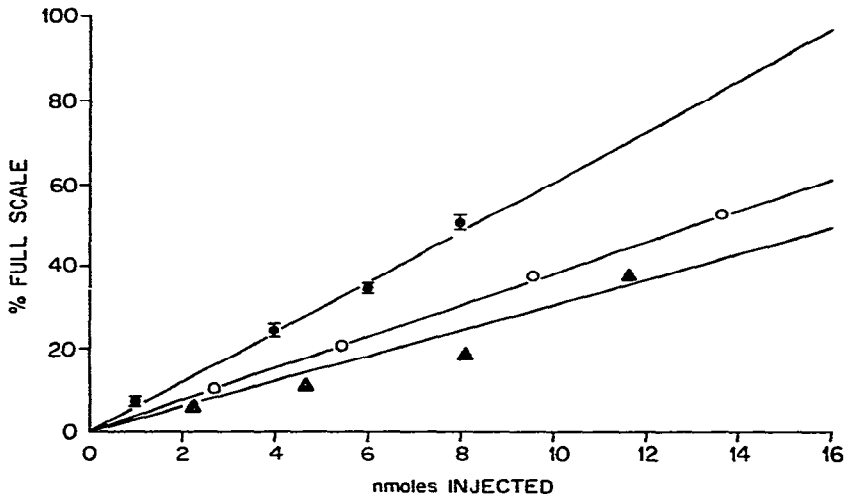

Fig. 3. Graphical illustration of the linearity of the NOD response for three compounds: sodium nitrite (●), nitrosomethylurethane (○), nitrosobutylurea (▲).

The NOD as described is specific for nitrosamides, nitrosoureas, nitrosourethane, nitrosoguanidines, and alkyl nitrites. All of these compounds contain relatively labile N–NO bonds. Alkyl nitrites release free nitrite in the presence of dilute acid at room temperature, while the other compounds require brief heating at 90°. The residence time in the heating bath was kept short to minimize analysis time and band spreading. Peaks on the colorimeter were, nevertheless, about 1/2 min wider than the corresponding peak on the HPLC. A baseline separation of 2 min on the HPLC was required to produce a baseline separation on the NOD.

All compounds tested eluted within a reasonable time (no more than 15 min) with appropriate choice of solvent composition. Fig. 4 shows elution of a mixture of methomyl and N-nitrosomethomyl, giving two peaks on the HPLC, as expected, and only one peak on the NOD. The usual solvent of choice was *n*-propanol–water (1 : 4) for carbamate pesticides and their nitroso derivatives. *n*-Propanol–water (1 : 19) was the solvent of choice for most other compounds tested. (This solvent mixture often resolved the *syn*- and *anti*-isomers of the nitroso compounds.)

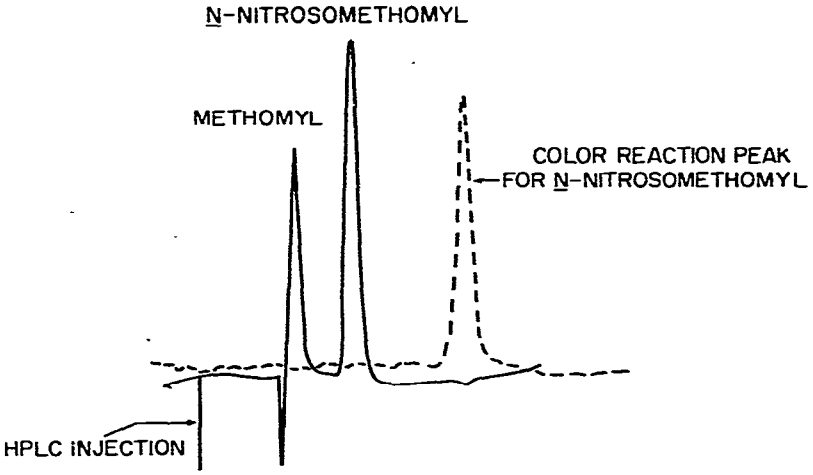

Fig. 4. HPLC and NOD traces for analysis of a mixture of methomyl and N-nitrosomethyomyl. HPLC conditions: 20% *n*-propanol–water, flow-rate 1.5 ml/min. ———, HPLC trace; — — —, NOD trace.

The limit of sensitivity for sodium nitrite is 35 ng injected, or 0.5 nmoles (*i.e.*, at $S/N = 3$). This should be the theoretical maximum sensitivity of the NOD.

To increase the versatility of the NOD, two valves and an injector were installed between the HPLC and the NOD to allow for direct injection of a sample into the NOD flow system (see Fig. 5). A 6-port distributing valve (Altex) immediately downstream from the HPLC allows the direction of the HPLC effluent into the NOD or to waste, as well as allowing for future applications. A sample passing directly into the NOD then passes through a 4-port rotary valve (Pharmacia) which allows a make-up solution of propanol–water to be pumped through the flow system while venting the HPLC effluent. The latter situation would arise during the direct analysis of a nitrosation mixture without work-up. The excess nitrite would seriously disturb the NOD and simply venting the HPLC effluent starves the NOD. A septum injector between the 4-port rotary valve and the NOD allows direct injection of samples for nitrite analysis, bypassing the HPLC.

It is also possible to analyze for sodium nitrite as part of a mixture or by itself using this system. A solution containing sodium nitrite can be injected directly into the HPLC, and the sodium nitrite will elute in the void volume.

The detector is particularly useful for following the course of nitrosation reactions. Minimal work-up is required, and the relative amounts of nitrosated and unnitrosated compound are easily determined.

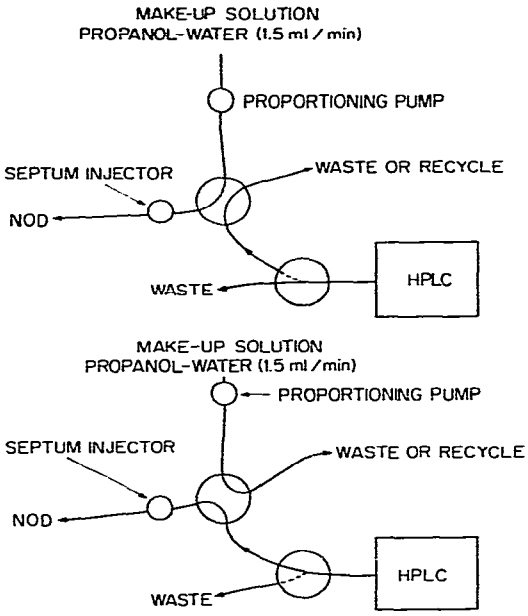

Fig. 5. Valving system used between the HPLC and the NOD. Top: use of NOD for nitrite or group analysis (without HPLC). Bottom: configuration of valves when using HPLC with the NOD.

TABLE II

EFFICIENCIES OF REACTION REPRESENTATIVE NITROSO COMPOUNDS RELATIVE TO SODIUM NITRITE

Compound	*Efficiency (%)*	*Standard deviation (%)*	*Number of points*
Nitrosomethylurea	93*	± 9	3
Trimethylnitrosourea	108*	± 2	4
N-Ethyl-N'-nitro-N-nitrosoguanidine	87*	± 6	3
Nitrosomethylurethane	105**	± 10	4
Nitrosoethylurea	95**	± 2	4

* HPLC eluent *n*-propanol–water (1:1).
** HPLC eluent *n*-propanol–water (1:19).

The system is clearly potentially useful for identification of nitrosocarbamates, *etc.*, in environmental samples. Extraction and subsequent concentration to obtain detectable levels of compound would, of course, be necessary. However, there need be little concern about interference from most biological substances. Fan and Tannenbaum[5] found that a wide variety of substances, including salts of organic and inorganic acids, sugars, nucleosides, bases, and amino acids, did not interfere with the Griess reagent.

It is of course, necessary to remove protein from biological samples before injection onto the HPLC column. Also, all particulate matter should be removed by sample filtration through a 0.5-μ filter.

ACKNOWLEDGEMENTS

We wish to thank Dr. W. Lijinsky for his interest and help with our work, and for providing many of the compounds tested. We also wish to thank Dr. James Epler and Dr. Mayo Uziel for many helpful discussions.

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