Journal of Chromatography, 124 (1976) 105–110

CHROM. 9292

Note

Liquid chromatography of N-nitrosoamino acids and their *syn* and *anti* conformers

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(Received March 9th, 1976)

High-pressure liquid chromatography (HPLC) has been used to separate volatile and nonvolatile nitrosamines[1–3] and their derivatives[4]. Recently, we reported the separation of the *syn* and *anti* conformers of several N-nitroso derivatives on various columns and under different conditions.

Both N-nitrosoproline (NPro) and N-nitrososarcosine (NSar) have important environmental implications. NPro has been reported in raw bacon at the parts per million level[5]. It has also been reported to decarboxylate in model systems of bacon under conditions similar to those commonly used for frying bacon[6–8]. The decarboxylated product, N-nitrosopyrrolidine (NPyr), has been shown to be carcinogenic in animal feeding studies[9,10]. NSar is a weak carcinogen in the rat[9] but more potent in the mouse[11]. It can result from the reaction of nitrite and creatine, a compound normally found in muscle tissue of many vertebrates[12]. NSar could also decarboxylate upon heating to form the potent carcinogen, N-nitrosodimethylamine.

Because it is difficult to analyze and quantitate two conformer peaks for each nitrosoamino acid by HPLC, it would be desirable to find columns and conditions that eliminate the *syn* and *anti* conformer effects, yet separate the important compounds.

This article describes several columns and conditions that gave *syn* and *anti* conformer separations as well as single-peak separations for NPro and NSar. It also describes briefly the elution characteristics on reversed-phase columns of other nonvolatile N-nitroso compounds, such as N-nitrosohydroxyproline (NHyPro) and N-methyl-N-nitrosourea (NMU), as well as the volatile nitrosamines NPyr and N-nitrosodiethylamine (DEN).

EXPERIMENTAL

NPro, NSar, and NHyPro were synthesized by the nitrosation of the respective amino acids (Sigma, St. Louis, Mo., U.S.A.) as described by Hansen *et al.*[13]. NMU, NPyr, and DEN were obtained from commercial sources.

HPLC measurements were performed on a Model ALC 202 liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) equipped with a 254-nm fixed-wavelength detector.

Chromatography of the N-nitroso compounds was studied on any one of four columns: (1) 60.96 cm × 2.1 mm I.D. Bondapak C_{18}/Corasil (Waters Assoc.), (2) 60.96 cm × 2.1 mm I.D. Bondapak C_{18}/Porasil B (Waters Assoc.), (3) 60.96 cm × 2.1 mm I.D. Bondapak Phenyl/Porasil B (Waters Assoc.), and (4) 75 cm × 2.1 mm I.D. Pellidon (Reeve Angel & Co., Clifton, N.J., U.S.A.). The solvent used with reversed-phase column packings was 1% Na_2HPO_4, adjusted to pH 2, 3, or 4 with concentrated phosphoric acid. The solvent used for the Pellidon polyamide packing was 0.1% acetic acid in tetrahydrofuran. The flow-rate 1 ml/min and the column temperature 25°.

RESULTS AND DISCUSSION

In a previous paper[3] we reported the partial separation of the *syn* and *anti* conformers of NPro and NSar on anion-exchange and reversed-phase HPLC columns. In our search for different packing materials and solvents to separate nonvolatile nitrosamines we found a system that gave a baseline separation of the *syn* and *anti* conformers of NPro (Fig. 1). This separation was achieved on a pellicular polyamide packing with an eluent of 0.1% acetic acid in tetrahydrofuran. To confirm the presence of *syn* and *anti* conformers, a freshly prepared solution of NPro was injected onto the column; it gave only one peak, which corresponded to the *syn* conformer in which NPro preferentially crystallizes.

The mixture of the nitrosoamino acids NPro and NSar produced a single peak when chromatographed on a Bondapak C_{18}/Corasil column using a 1% solution of

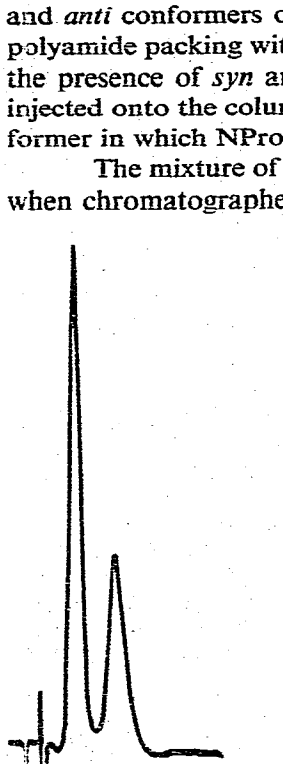

Fig. 1. Separation of the *syn* and *anti* conformers of N-nitrosoproline. Column, 75 cm × 2.1 mm I.D. Pellidon; mobile phase, 0.1% acetic acid in tetrahydrofuran; flow-rate, 1.0 ml/min.

Na_2HPO_4 (pH 8.2) as eluent; both compounds eluted just after the solvent front. However, when the pH of the eluent was lowered to 3.0 with H_3PO_4, NPro was retained longer on the column, and separation of the nitrosoamino acids was achieved. The resolution of the single peaks for NPro and NSar was improved by using a column packed with Bondapak C_{18}/Porasil B, a reversed-phase packing material with a larger surface area and greater capacity than C_{18}/Corasil (Fig. 2). Table I shows the chromatographic parameters for the separation of NPro and NSar on each column. The separation of the nitrosoamino acids was achieved only after a wetting agent or

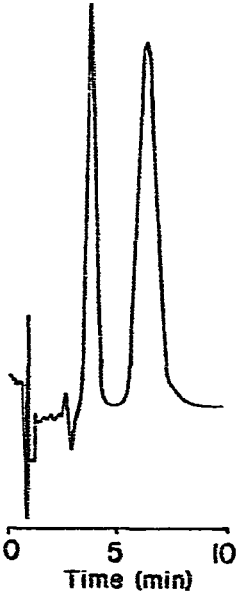

Fig. 2. Separation of NPro and NSar on Bondapak C_{18}/Porasil B (1% phosphate buffer, pH 3). Column: 60.96 cm × 2.1 mm I.D.

TABLE I

CHROMATOGRAPHIC PARAMETERS OF NSar AND NPro ON BONDAPAK C_{18}/CORASIL AND BONDAPAK C_{18}/PORASIL B

Abbreviations: N = Total number of plates; k' = capacity factor, $(t_R - t_0)/t_0$; $\bar{H}$ (mm) = HETP; R_s = resolution.

Column	*Parameter*	*Nitrosamine*	
		NSar	*NPro*
C_{18}/Corasil	N	256	288
	k'	0.22	0.89
	$\bar{H}$ (mm)	2.38	2.12
	R_s	1.78	
C_{18}/Porasil B	N	224	176
	k'	0.5	1.8
	$\bar{H}$ (mm)	2.72	3.47
	R_s	2.08	

plasticizer, such as acetonitrile or tetrahydrofuran, had been introduced onto the column in a one-time injection of approximately 100 μl. This procedure allowed partition processes to take place. However, if any amount of acetonitrile was added to the aqueous eluent, the nitrosoamino acids eluted at the solvent front.

Changing to a column containing Bondapak Phenyl/Porasil B, a more polar reversed-phase packing, greatly increased the resolution between NPro and NSar when the same 1% Na_2HPO_4, pH 3, eluent was used. Again, an initial, single injection of a wetting agent was necessary. This packing material not only separated NPro and NSar from each other, but also partially separated the *syn* and *anti* conformers of each nitrosoamino acid (Fig. 3b). Figs. 3a and 3c show the separation of NPro and NSar when the pH is adjusted to 4.0 or 2.0, respectively. At both pH 3.0 and 4.0, the conformers of NPro and NSar were partially separated from each other, indicating a difference in partition coefficients of the two conformers between the aqueous solvent and the phenyl stationary phase.

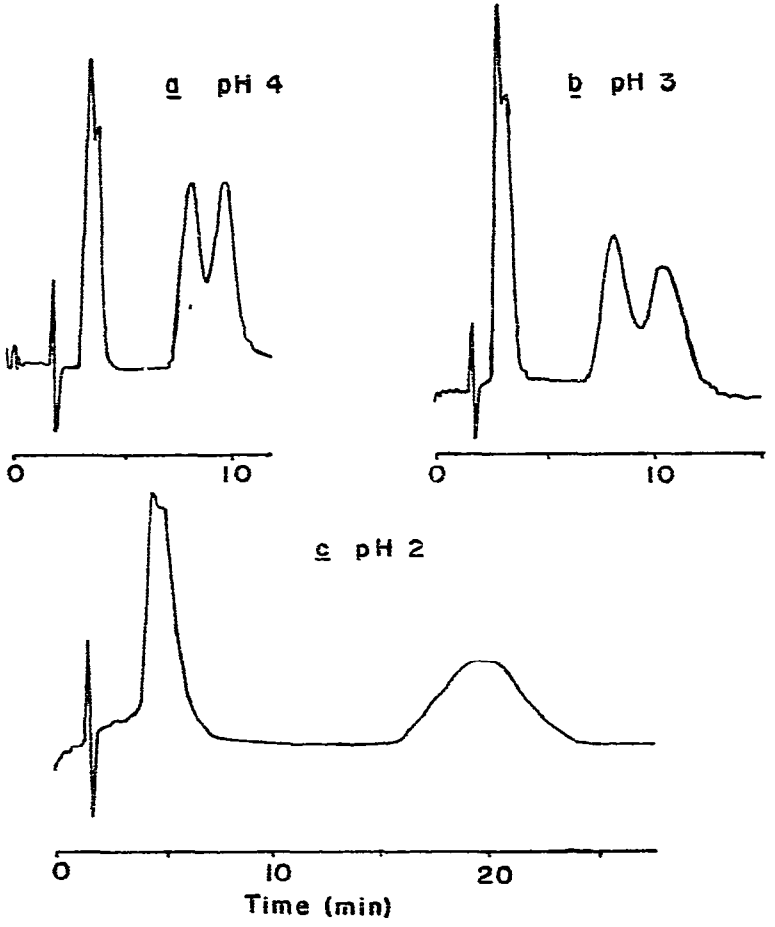

Fig. 3. Separation of NSar and NPro, and their respective *syn* and *anti* conformers. Column, 60.96 cm × 2.1 mm I.D. Bondapak Phenyl/Porasil B; mobile phase 1% phosphate buffer; variable pH.

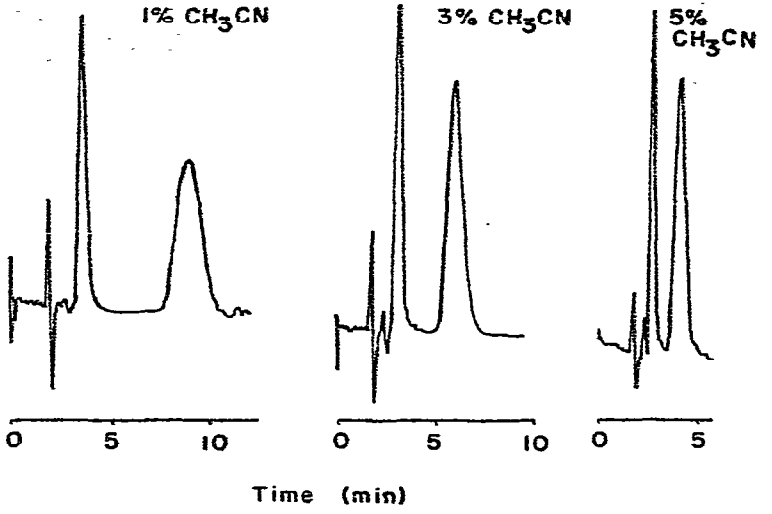

Fig. 4. Separation of NSar and NPro. Column 60.96 cm × 2.1 mm, I.D. Bondapak Phenyl/Porasil B; mobile phase: 1% phosphate buffer, pH 2; variable acetonitrile concentration.

At pH 2.0, the conformers of NSar separated slightly, but NPro was eluted as a broad single peak late in the chromatogram. The single peak of NPro was due either to an unsuccessful resolution of the *syn* and *anti* conformers because of the length of time in the column, or to identical partition coefficients for the conformers of NPro at pH 2.0 (pK_a of NPro = 3.0). The three chromatograms in Fig. 3 clearly indicate the increasing affinity of both the conformers and the individual nitrosoamino acids for the aqueous mobile phase with decreasing pH values.

When as little as 1% acetonitrile was added to the eluting solvent (1% Na_2HPO_4, pH 2.0), the slight *syn* and *anti* conformer separation disappeared for NSar, and the retention time was greatly reduced for NPro. The chromatograms in Fig. 4 show the effects on the separation of NPro from NSar of adding various amounts of acetonitrile to the eluent; Table II shows the differences in chromatographic parameters with varying amounts of acetonitrile.

When 1% acetonitrile was added to the eluent at pH 3, the partial separation

TABLE II

CHROMATOGRAPHIC PARAMETERS OF NPro AND NSar ON BONDAPAK PHENYL/PORASIL B

Nitrosoamino acid	*Per cent acetonitrile*	*N*	*k'*	*$\bar{H}$ (mm)*	*R_s*
NSar	1	285	1.1	2.14	3.33
	3	336	0.6	1.82	2.60
	5	320	0.35	1.9	1.87
NPro	1	240	4.15	2.54	
	3	240	2.1	2.54	
	5	338	1.1	2.56	

of the *syn* and *anti* conformers for NSar disappeared; the conformers of NPro were also less well resolved, indicating that low pH was necessary to suppress ionization of the carboxyl group.

The fact that the *syn* and *anti* conformers of NPro and NSar can be separated from each other by adjusting the pH and the acetonitrile concentration of the eluent makes this chromatographic separation useful as a possible confirmation step for the presence of these nitrosamines.

Further studies with different nonvolatile N-nitroso compounds indicate that Bondapak Phenyl/Porasil B- and/or Bondapak C_{18}/Porasil B-packed columns can be very versatile for separations of N-nitroso compounds. For example, NHyPro, NMU, NSar, and NPro are readily separated on these columns. Similarly, the homologous series of N-nitrosoureas would be expected to separate according to the work of Heyns and Roper[2], who separated N-nitrosoureas and N-nitrosourethanes on a Bondapak C_{18}/Corasil column using water–acetonitrile. The advantage of using Porasil B reversed-phase packings is in their greater surface area and capacity. The resultant increased retention of the compounds allows manipulation of other variables to achieve the desired separation. It should also be noted that the less polar, more volatile N-nitroso compounds NPyr and DEN do not elute from these reversed-phase packings with purely aqueous eluents.

The results presented here also show how aqueous solutions can be used to separate ionic compounds on reversed-phase packing materials using pH to control the degree of ionization. The separation of other organic acids and bases should also be possible under similar circumstances.

ACKNOWLEDGEMENT

This research was supported by Public Health Service Contract NO1-CP33315 with the Division of Cancer Cause and Prevention, National Cancer Institute.

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