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CHROMATOGRAPHIC SEPARATION OF CONFORMERS OF SUBSTITUTED ASYMMETRIC NITROSAMINES

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SUMMARY

The *syn* and *anti* conformers of N-nitrosoproline, N-nitrososarcosine and N-nitroso-2-(ethylamino)-ethanol, have been separated by liquid chromatography. These conformers result from hindered rotation about the N–N bond. Separation was achieved using adsorption, reversed-phase, and ion-exchange modes. For the nitroso-amino acids, a shift in the equilibrium conformer concentration was observed with changes in pH.

INTRODUCTION

Nitrosamines have recently been shown to be an important class of environmental carcinogens[1–4]. Many reports have been published dealing with numerous types of chromatographic separations[5–10], physical and spectral properties[11] and methods of quantitation[5,7–13]. However, there is only one report of a separation of nitrosamine conformers by thin-layer chromatography (TLC)[14]. In our work with substituted asymmetric nitrosamines, we have achieved separation of *syn* and *anti* conformers using high-pressure liquid chromatography (HPLC).

The conformers in nitrosamines occur because of the contribution of polar resonance forms[15]. The resonance leads to a partial double bond character of the N–N bond due to delocalization of the lone pair of nitrogen electrons with the hetero π electron system. This results in a barrier to free rotation of the nitroso group (23 kcal/mole for N-nitrosodimethylamine[16]). Consequently, the nitroso-amino group assumes an essentially planar conformation in which the oxygen atom is *syn* to one substituent and *anti* to the other (Fig. 1). When $R_1 \neq R_2$, two isomeric conformations are possible, and can generally be distinguished by nuclear magnetic resonance (NMR) spectroscopy[16–22]. This paper describes liquid chromatographic data obtained with conformers of N-nitroso-2-(ethylamino)-ethanol (NEE), N-nitrosoproline (NP), and N-nitrososarcosine (NS).

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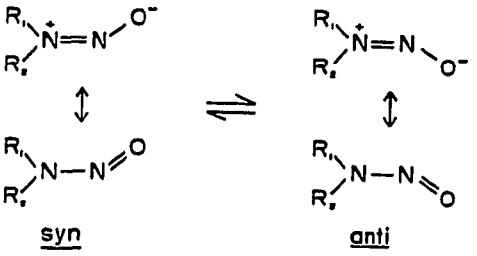

Fig. 1. Polar resonance forms of nitrosamines. The designations *syn* and *anti* refer to the position of the nitroso group with respect to R_1.

EXPERIMENTAL

NEE was synthesized by nitrosation of 2-(ethylamino)-ethanol (Aldrich, Milwaukee, Wisc., U.S.A.) as described by Druckrey *et al.*[1]. NP and NS were synthesized by nitrosation of proline and sarcosine (Sigma, St. Louis, Mo., U.S.A.), respectively, using a procedure[23] modified from that of Lijinsky *et al.*[24].

HPLC measurements were performed on a Waters Ass. Model ALC 202 liquid chromatograph (Milford, Mass., U.S.A.) equipped with a 254-nm fixed-wavelength ultraviolet detector. Infrared (IR) measurements were performed on a Perkin-Elmer 257 grating IR spectrophotometer (Norwalk, Conn., U.S.A.).

Chromatography of NEE was studied on a 4-ft. × 2.1-mm-I.D. Corasil II column (Waters Ass.). The eluting solvent was nanograde dichloromethane (Mallinckrodt, St. Louis, Mo., U.S.A.). Chromatography of NP was studied on a 2-ft. × 2.1-mm-I.D. Sepcote 7AN anion-exchange column (Separations Technology, Cambridge, Mass., U.S.A.) with 0.075 *M* ammonium phosphate adjusted to pH 3.5 as eluent. NP and NS were also studied on a Bondapak C_{18}/Porasil (Waters Ass.) reversed-phase column with an acetonitrile–water (95:5) mixture as solvent. TLC was performed on a 2-in. × 8-in. glass plate coated with a 0.5-mm layer of silica gel G (Merck, Darmstadt, G.F.R.). The developing solvent was 95% ethanol–benzene–water (4:1:1).

NEE was dissolved in dichloromethane and allowed to stand at room temperature for 48 h. Sufficient NEE (25 μl of a 20 mg/ml solution) was injected onto the Corasil II column to give full-scale deflection at the lowest sensitivity (64×) and each of the two peaks was collected at the exit of the detector as it eluted from the column. After various time periods a small volume (40 μl) of each of the collected sample peaks was re-injected onto the column.

Crystalline NP was dissolved in distilled water or 0.075 *M* ammonium phosphate, adjusted to the required pH and allowed to stand at room temperature for 48 h. A sample of NP (50 μl of a 1 mg/ml solution) was injected onto the column to give a full-scale deflection on the chart paper at 64×. The two peaks of the NP chromatograms did not have a baseline separation so the leading edge of the first peak and the tailing edge of the second peak were collected for the equilibration study.

RESULTS AND DISCUSSION

The chromatogram of a sample of NEE which stood at room temperature for

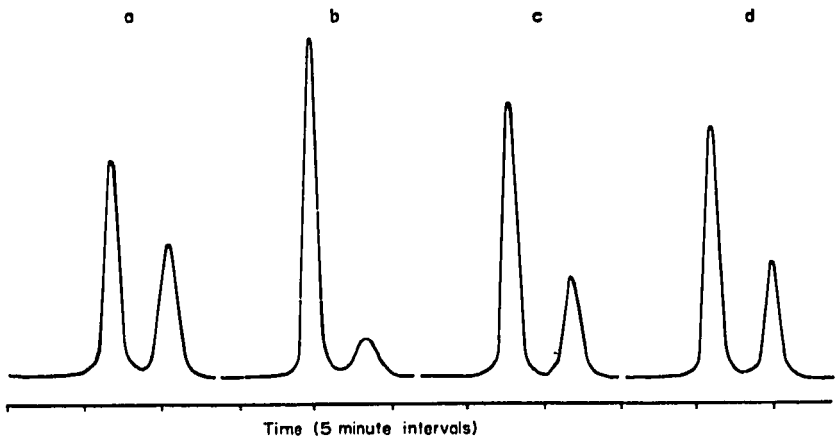

Fig. 2. Liquid chromatographic separation of the *syn* and *anti* conformers of NEE. (a) Equilibrium mixture. The first peak was collected for subsequent injections. (b) 40 min after collection. (c) 100 min after collection. (d) 230 min after collection. Column, 4 ft. × 2.1 mm I.D. Corasil II; mobile phase, dichloromethane, 2.1 ml/min.

48 h in dichloromethane is shown in Fig. 2a. The first of the two peaks eluting from the Corasil II column was trapped and portions were re-injected onto the column at various time periods. Figs. 2b–d show the gradual appearance of a new peak which had the same retention time as the second peak in the original NEE chromatogram. After 7 h, the ratio of the peak areas of the re-injected sample was the same as the original equilibrated sample (peak 1 : peak 2 = 51 : 49). The half-life of this transformation was 90 min. In a similar experiment, peak 2 was trapped and portions re-injected onto the column. In this case, a new peak appeared with the same retention time as the first peak in the original chromatogram. After 7 h, the ratio of peak areas was the same as the original equilibrated sample and the half-life of the transformation was again 90 min. IR spectra of the two peaks taken after trapping them at the detector exit were identical. This evidence suggests that the two components separated by liquid chromatography were the *syn* and *anti* conformers of NEE.

Anion-exchange chromatography of NP, freshly dissolved in 0.075 *M* ammonium phosphate adjusted to pH 3.5, showed a single peak with a retention time of 28 min. When the same material was allowed to equilibrate in the same solvent at room temperature for 48 h, however, two peaks with roughly equal areas were detected (Fig. 3). Similar to NEE, when each peak was trapped individually and portions were re-injected onto the column at various time periods, a gradual appearance of the respective second peak was observed. Fig. 4 shows the time course for the equilibration of the components of NP ($t_{\frac{1}{2}} = 4$ h).

Our finding of a single peak with freshly dissolved NP is consistent with the observation that NP crystallizes preferentially with the nitroso group *syn* to the carboxyl group[24]. Lijinsky *et al.*[24] detected only one conformer by NMR 4 min after dissolving NP in pyridine and assigned the spectrum to the *syn* form by chemical shift arguments. Thus, in our chromatogram for NP, peak 1 would correspond to the *syn* form and peak 2 to the *anti* form.

In another experiment, NP was allowed to equilibrate at room temperature

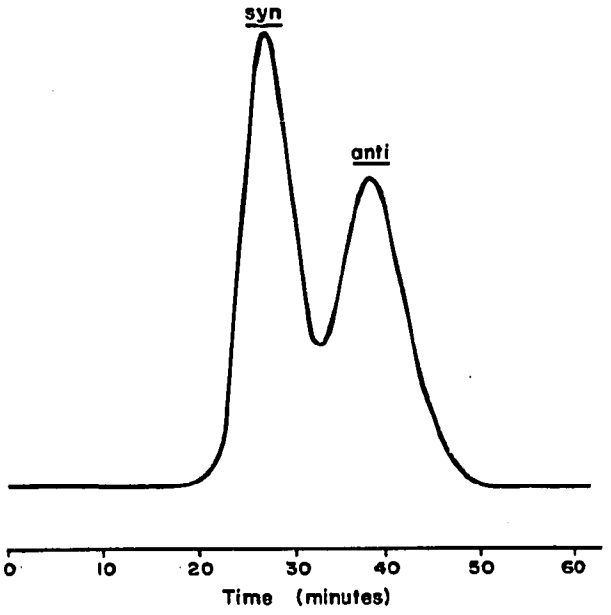

Fig. 3. Liquid chromatographic separation of the *syn* and *anti* conformers of NP. Column, 2 ft. × 2.1 mm I.D. Sepcote 7AN strong anion-exchange resin; mobile phase, 0.075 *M* ammonium phosphate, pH 3.5, 0.7 ml/min.

in solutions at different pH levels. Table I shows that the equilibrium constant for the interconversion of NP conformers remained essentially unchanged between pH 8.0 and 3.5, but the *syn* conformer increased in concentration at lower pH levels. This shift in equilibrium may be explained by protonation of the carboxyl group which would decrease electrostatic interactions between the ionized carboxyl and the electronegative nitroso group and would decrease the degree of solvation of the carboxyl group and hence its steric size. Protonation would also favor hydrogen bond interaction between the carboxyl and nitroso groups, which would increase the stability of the *syn* form relative to the *anti*. A pK_a value, determined to be 3.0 for nitrosoproline[24], is consistent with our data.

HPLC of NS showed a partial separation of *syn* and *anti* conformer peaks using the same column condition as for NP. The conformer peaks of NS had the same retention time as the corresponding conformer peaks of NP. Using a reversed-phase C_{18}/Porasil column with acetonitrile–water (95:5) as eluent, *syn* and *anti* conformers of NP and NS were partially resolved, but again the column was not able to separate the nitroso-amino acids from each other. Increasing the column temperature to 50° greatly decreased the resolution of the conformers for both NP and NS.

A complete separation of the conformers of NP using analytical TLC on silica gel was demonstrated. The R_F values were 0.57 and 0.50 for the *syn* and *anti* conformers when the developing solvent was 95% ethanol–benzene–water (4:1:1). The only other report of a chromatographic separation of the conformers of nitrosamines is by Mannschreck *et al.*[14]. They were able to separate the conformers of N-nitroso-N-benzyl-2,6-dimethylaniline ($t_{\frac{1}{2}}$ = 86 min) by two-dimensional TLC using benzene twice as the developing solvent. They were also able to obtain the conformers of N-

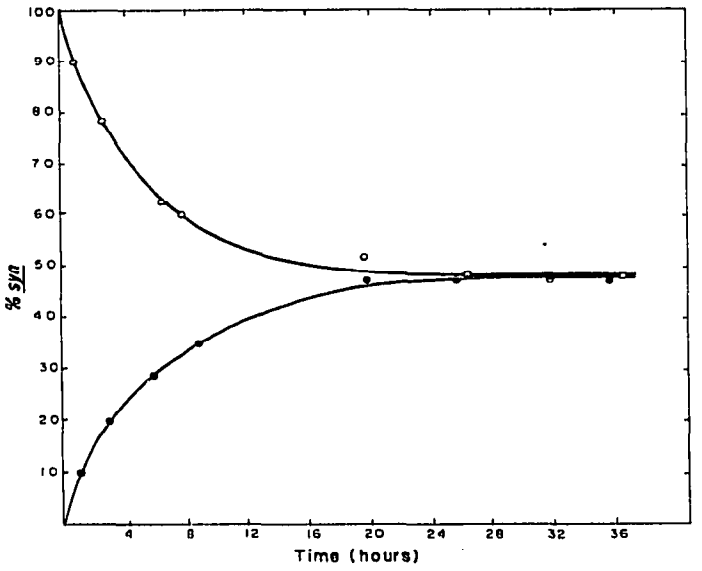

Fig. 4. Time course for equilibration of NP conformers. ●, Equilibration of peak 1 (*syn* conformer) after collection and reinjection of peak 2; ○, equilibration of peak 1 (*syn* conformer) after collection and reinjection of peak 1.

nitroso-N-isopropylbenzylamine at 75% and 94% purity for the *syn* and *anti* forms, respectively, through crystallization[14]. However, no details of their method were provided.

Although HPLC has only been recently introduced into the field of nitrosamine analysis by our laboratory[10] and by Cox[9], separation of individual conformers was not observed in these studies since only symmetrical nitrosamines or derivatized products were used. A liquid chromatogram obtained from extracts of food samples spiked with N-nitrosomorpholine or N-nitrosodimethylamine showed only a single peak for each nitrosamine. In his analytical procedure Cox[9] reduced the nitrosamines to their respective amines and formed 2,4-dinitrophenyl derivatives which then chromatographed as single peaks. Numerous gas chromatographic separations of nitrosamines

TABLE I

VARIATION OF EQUILIBRIUM CONSTANT WITH pH FOR THE INTERCONVERSION OF THE *Syn* AND *Anti* CONFORMERS OF NP

pH	$K_{(syn \rightleftarrows anti)}$
1.5	0.72
2.0	0.69
2.4	0.79
2.9	0.96
3.5	1.04
4.0	1.13
5.3	1.08
6.0	1.06
7.0	1.04
8.0	1.08

have been reported[7,11,25–29]. In no case, however, were *syn* and *anti* conformers separated. We have shown that elevated temperatures in HPLC decreased the resolution of the *syn* and *anti* forms. The higher temperatures utilized in gas chromatography would increase the rate of interconversion of the two conformers to the point where the half-life of each species is short compared to the residence time on the column. Therefore, only one peak is observed under these conditions.

Although several articles have described physico-chemical properties of nitrosamine conformers as determined by NMR, this is the first report about the separation of conformers of substituted asymmetric nitrosamines by HPLC using a variety of separation modes. Our observations are particularly pertinent to the recent interest in the development of analytical methods for carcinogenic nitrosamines which occur in the environment.

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