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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATIONS OF NITROSAMINES

III. CONFORMERS OF N-NITROSAMINO ACIDS

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SUMMARY

The separation of a selected group of N-nitrosamino acids and their *syn* and *anti* conformers by high-performance liquid chromatography using an α-cyclodextrin bonded silica gel column and a mobile phase of acetonitrile–triethylammonium acetate was achieved. The effects of mobile phase pH and concentration of acetonitrile on resolution and elution times are also reported.

INTRODUCTION

The separations of cyclic[1] and acyclic[2] nitrosamines by high-performance liquid chromatography (HPLC) using β-cyclodextrin and C_{18} bonded silica columns were reported. This research deals with separation of N-nitrosamino acids and their *syn* and *anti* conformers. N-nitrosamino acids excreted in the feces and urine are used as an index of endogenous nitrosation. This is based on the findings that N-nitrosamino acids such as nitrosoproline (NPRO), nitrosohydroxyproline (NHPRO) and nitrososarcosine (NSAR), when administered orally to rats, are excreted unchanged almost quantitatively in the urine and feces[3]. The reaction of nitrite and creatinine forms NSAR, and NPRO can result from the reaction of the amino acid proline under nitrosating conditions[4]. Cyteine can form nitrosothiazolidine carboxylic acid (NTCA) in the presence of nitrite and formaldehyde under nitrosating conditions[4].

Determination of N-nitrosamino acids by gas chromatography (GC) has been reported after derivatization of the carboxyl groups by methylation[5,6] or silylation[7]. Another GC procedure was developed whereby the carboxyl group was esterified with diazomethane, and the nitroso group was oxidized with peroxytrifluoroacetic acid to give N-nitrosamino acid methyl esters. The hydroxyl group of NHPRO methylester was acylated with trifluoroacetic anhydride. The derivatives were then analyzed by

a GC utilizing an electron-capture detector[8]. In our laboratory, a GC equipped with a thermal energy analyzer (TEA) is routinely used for the determination of nitrosamines and nitrosamino acids.

Although GC with electron-capture detection (ECD) or TEA is a sensitive technique for analyzing nitrosamines and their derivatives, it may not be the best technique for the resolution of the *syn* and *anti* conformers since the analysis is carried out at above room temperature. Cryogenic thin-layer chromatography (CTLC) was successfully used for the separation of the *syn* and *anti* conformers of some dinitrosopiperazines[9]. Also, HPLC, using β-cyclodextrin and reversed-phase C_{18} bonded silica gel columns at room temperature, was used to separate some acyclic nitrosamines and their *syn* and *anti* conformers[2]. Iwaoka and Tennenbaum[10] were able to separate the *syn* and *anti* conformers of NPRO by HPLC using a pellicular polyamide column with an eluent of 0.1% acetic acid in tetrahydrofuran. However, they were only partially, but not quantitatively, able to separate the *syn* and *anti* conformers of NSAR[10]. Walters *et al.*[11] used HPLC to separate five N-nitrosamino acids from each other but not their conformers.

This study is an extention of our previous studies[1,2] into the use of cyclodextrin bonded packings for the separation of nitrosamines. The present study is an evaluation of the α-cyclodextrin bonded silica gel HPLC column for the separation of a group of nitrosamino acids and, where applicable, their *syn* and *anti* conformers. Also the effect of pH and percent organic modifier on resolution and retention will be studied. For these purposes, a group of five- and six-membered ring nitrosamino acids and NSAR were selected, the structures of which are shown in Fig.1.

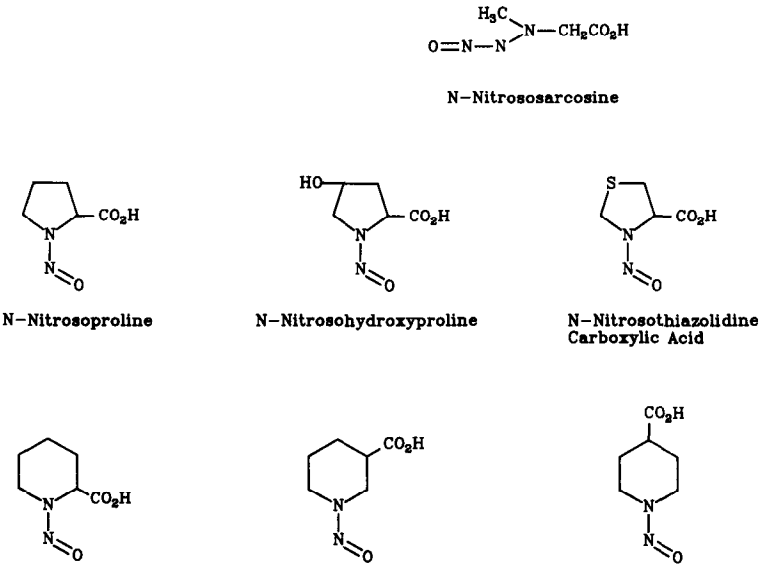


Fig. 1. Structural formulae of nitroso compounds used in this study.

EXPERIMENTAL

Materials

The nitrosamino acids used in this study, NSAR, NPRO, NHPRO, NTCA, N-nitrosopipecolinic acid (NPPC), N-nitrosonipecotic acid (NNPC) and N-nitrosoisonipecotic acid (NINPC), were synthesized in house by the method of Lijinsky *et al.*[12] and their structures were confirmed by elemental analysis, mass spectrometry and nuclear magnetic resonance. Acetonitrile was glass-distilled UV grade (Burdick & Jackson). Water was deionized glass distilled. The 5-μm spherical prepacked α-cyclodextrin (cyclobond III) bonded silica gel columns (150 × 4.6 mm I.D., and 250 × 4.6 mm I.D.) were purchased from Advanced Separations Technologies (Whippany, NJ, U.S.A.).

Apparatus

A Hewlett-Packard Model 1090 liquid chromatograph equipped with a photodiode array detector, an automatic injector, a strip chart recorder, a Hewlett-Packard Model 3392A integrator and a Hewlett-Packard Model 85 computer/controller was used.

Procedure

Solutions were prepared in water to contain approximately 0.5 μg/μl. A 10-μl volume of the solution was injected, unless specified. The mobile phase was made of acetonitrile–triethylammonium acetate (TEAA), which was filtered and degassed before use and maintained under helium throughout the experiment. Mobile phase flow-rate was 1.5 ml/min. Absorption was monitored at 238 nm.

The TEAA solution (0.01 *M*) was prepared by adding 1.4 ml triethylamine to 1.0 l of water and then titrating with acetic acid to the required pH. Throughout this manuscript TEAA will refer to a 0.01 *M* TEAA solution. pH measurements were made using a Fisher Accumet brand pH meter Model 750.

RESULTS AND DISCUSSION

In two previous papers we reported the separation of cyclic[1] and acyclic nitrosamines and some of their conformers[2] by HPLC. This work deals mainly with the separation of the *syn* and *anti* conformers of nitrosamino acids with emphasis on NSAR, NPRO, NHPRO and NTCA, which are produced endogenously.

Fig. 2 shows the separation of the *syn* and *anti* conformers of NTCA, NSAR, NPRO and NHPRO, using an α-bonded cyclodextrin column and a mobile phase of acetonitrile–TEAA (90:10, v/v) at pH 5. The figure shows base line resolution of the conformers of NHPRO, NPRO and NSAR and a good resolution, which can be quantified, of the NTCA conformers. The retention times of each pair are listed in Table I. The *syn* and *anti* conformers were determined by nuclear magnetic resonance.

The *syn* and *anti* conformers of a mixture of NPRO, NHPRO and NSAR were resolved using the same column and mobile phase, but a pH of 2.5, Fig. 3. It is not recommended to use the α-cyclodextrin column for long periods of time with low pH mobile phases. This low pH was used in order to study the effect of pH on resolution, peak position, and elution order as will be discussed later. However, Fig. 3 shows

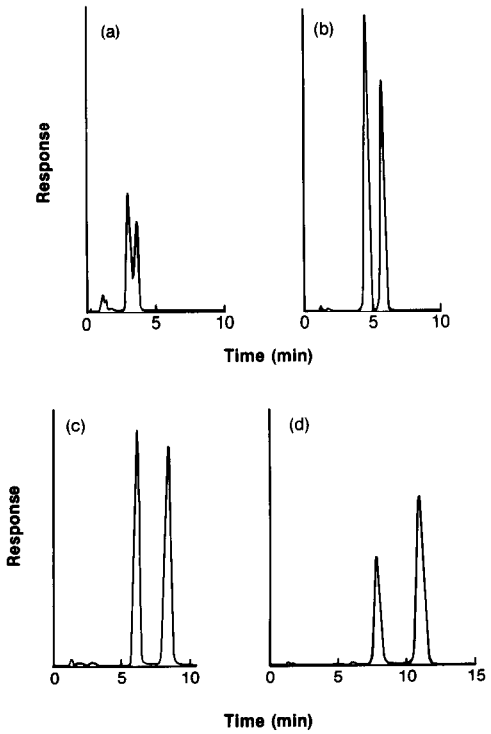


Fig. 2. Chromatograms of the separation of the *syn* and *anti* conformers of (a) NTCA, (b) NSAR, (c) NPRO and (d) NHPRO using a 150 × 4.6 mm α-cyclodextrin bonded column, 5 μm spherical, and a mobile phase of acetonitrile–0.01 *M* TEAA, pH 5 (90:10) at a flow-rate of 1.5 ml/min. Detection was carried out at 238 nm.

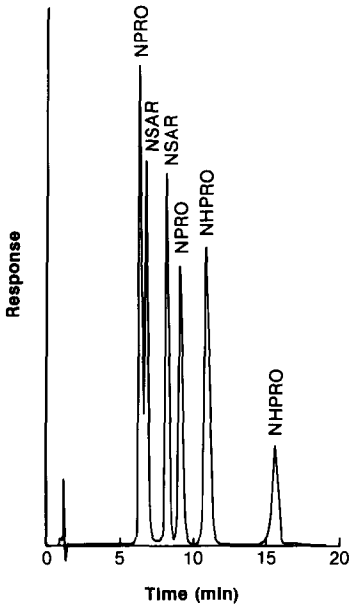
a good resolution of the six conformers in the mixture. Fig. 4 shows the separation of the conformers of the five-membered ring nitrosamino acids, NTCA, NPRO and NHPRO, using a mobile phase of acetonitrile–TEAA (92:8, v/v) at pH 5. The results show a partial resolution of one of the conformers of each NPRO and NHPRO, and a quantitative resolution of the other conformers.

TABLE I

RETENTION TIMES OF THE *SYN* AND *ANTI* CONFORMERS OF NTCA, NSAR, NPRO AND NHPRO

Conditions: 15 cm × 4. mm α-cyclodextrin bonded column, and a mobile phase of acetonitrile–TEAA (90:10, v/v), pH 5, at a flow-rate of 1.5 ml/min.

Compound	*Retention time (min)*
NTCA	3.11, 3.69
NSAR	4.63, 5.74
NPRO	6.04, 8.29
NHPRO	7.80, 10.92



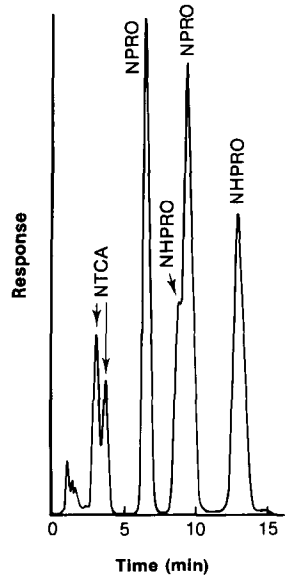


Fig. 3. Chromatogram of the separation of a mixture of NPRO, NSAR, and NHPRO. Conditions as in Fig. 2 except acetonitrile/0.01 *M* TEAA, pH 2.5 (80:20).

Fig. 4. Chromatogram of the separation of a mixture of five-membered ring N-nitrosamino acids. Conditions as in Fig. 2 except 92% acetonitrile.

Effect of mobile phase pH on the separation of NHPRO conformers

It was observed that the pH of the mobile phase affects not only the elution time of the nitrosamino acids but the resolution of the conformers and the shape of the peaks. For example, the *syn* and *anti* conformers of NHPRO shifted their elution positions when the pH was lowered from pH 4 to pH 3 to pH 2.75, Fig. 5. Two interesting phenomena are observed in Fig. 5: (i) at pH 4, (5a), the *anti* conformer of NHPRO eluted before the *syn* conformer. In contrast, at pH 3, (5b), the *anti* conformer eluted just after the *syn*, while the elution time of the *syn* conformer remained constant. (ii) When the pH was lowered to 2.75 (5c), the elution time of the *anti* conformer was about the same as it was at pH 3, but the *syn* conformer eluted earlier than it had at pH 3 and pH 4. This behavior may be due to the different pK_a's of these conformers. These phenomena may be used to an advantage in order to achieve the separation of other certain mixtures of nitrosamino acids and their conformers.

Six-membered ring nitrosamino acids

Three six-membered ring amino acids, NPPC, NNPC and NINPC, were used in this study. Only two of these, NPPC and NNPC, have *syn* and *anti* conformers. The separation of the three acids is shown in Fig. 6 using acetonitrile–TEAA (90:10, v/v) at pH 5. These compounds eluted quickly at lower pH's (results not shown). The conformers of these acids were resolved, although not base line, by using a mobile phase of acetonitrile–TEAA (70:30, v/v) at pH 5 as seen in Fig. 7. It is believed based on

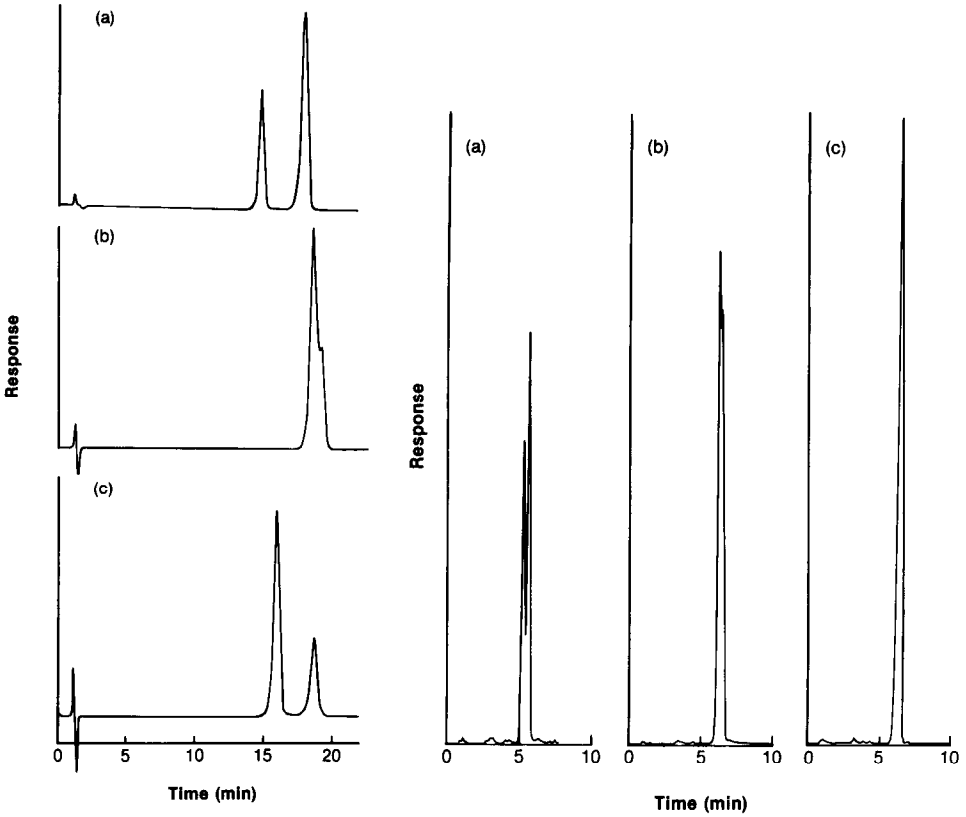


Fig. 5. Effect of pH on the separation of the *syn* and *anti* conformers of NHPRO on a 250 × 4.6 mm α-cyclodextrin column using a mobile phase of acetonitrile–0.01 *M* TEAA (80:20) at pH 4 (a), pH 3 (b) and pH 2.75 (c) and a flow-rate of 1.5 ml/min.

Fig. 6. Chromatogram of the separation of six-membered ring N-nitrosamino acids using a 150 × 4.6 mm α-cyclodextrin column and a mobile phase of acetonitrile–0.01 *M* TEAA, pH 5 (90:10) at a flow-rate of 1.5 ml/min.

previous experience[9] that lowering the temperature of analysis will result in the complete resolution of the conformers of NPPC and NNPC. This will be dealt with in a later publication studying the effect of temperature on the HPLC separation of nitrosamines and their conformers.

Effect of organic modifier

Acetonitrile was preferred to methanol because of less back pressure (viscosity) and the shapes of the peaks were more symmetrical when acetonitrile was used in the mobile phase. The greatest effect of the percent acetonitrile in the mobile phase on the retention of the nitrosamino acids was above 70%. Below that, the compounds eluted close to the solvent front, while above that, greater retention was observed (Table II). The results in Table II show some peculiarities; in most cases, retention times increased with an increase in the volume of acetonitrile (greater than 80%) in the mobile phase

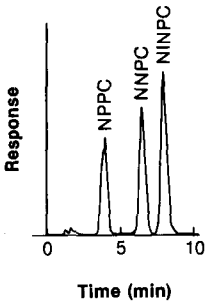


Fig. 7. Chromatograms of the separation of the *syn* and *anti* conformers of (a) NPPC and (b) NNPC. (c) Is a chromatogram of NINPC. Conditions are the same as in Fig. 6, except 70% acetonitrile.

under constant pH (NSAR, NPRO, NHPRO, NNPC and NINPC'); in other cases, the retention times decreased with an increase of the volume of acetonitrile up to −90% then incresed (NTCA and NPPC). This may be due to the fact that these nitrosamine acids have different pK_a values and also different solubilities. The relatively very long retention times at 95% acetonitrile are due to the fact that these N-nitrosamino acids are more soluble in water.

TABLE II

EFFECT OF PERCENTAGE ACETONITRILE IN THE MOBILE PHASE ON THE RETENTION OF N-NITROSAMINO ACIDS IN THIS STUDY

The conditions used were: 15 cm × 4.6 mm α-cyclodextrin bonded column, 5 μm spherical, and a mobile phase of acetonitrile–TEAA, pH 5, at a flow-rate of 1.5 ml/min.

Compound	t_R *(min)*				
	Acetonitrile (%)				
	60	*70*	*80*	*90*	*95*
NSAR	4.80	4.81	4.80	5.21	9.47
	5.35	5.48	5.91	6.49	12.48
NTCA	4.38	4.01	3.62	3.48	5.54
	4.78	4.45	4.19	4.18	7.15
NPRO	5.46	5.54	5.92	6.95	14.40
	6.32	6.63	7.18	9.64	21.72
NHPRO	5.22	5.71	6.57	9.06	21.37
	5.88	6.71	8.12	12.73	33.66
NPPC*		4.41		4.45	
	4.99	4.69	4.15	4.82	9.08
NNPC*	5.31	5.31	5.90	7.76	19.74
NINPC	5.40	5.62	6.37	10.06	27.57

* NPPC and NNPC, which have two conformers, rarely had their conformers separated under these conditions. Hence, only one elution time might be listed.

CONCLUSION

The results of this study show that an HPLC system equipped with an α-cyclodextrin bonded column and an isocratic mobile phase of acetonitrile–TEAA can be used to resolve the *syn* and *anti* conformers of NSAR, NPRO, NHPRO, NTCA, NNPC and partial separation of NNPC. It was found that at lower pH (2.5) the six-membered ring nitrosamino acids eluted quickly while they were retained longer at higher pH (5.0); good resolution of the conformers of the five-membered ring nitrosamino acids were resolved at both lower pH (2.5) and higher pH (5.0) mobile phase buffers. The results also show that, in most cases, longer retention times were observed, at the same mobile phase pH with an increase in the volume of acetonitrile in the mobile phase.

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