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GAS CHROMATOGRAPHIC SEPARATION OF HYDROXYLATED N-NITROSAMINES

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SUMMARY

Derivatives of six hydroxylated N-nitrosamines were prepared by acylation, trifluoroacylation, trimethylsilylation and methylation, and the volatilities and sensitivities of these derivatives on gas chromatographic detection were compared. All six nitrosamines were successfully derivatized by either acylation or trimethylsilylation. The acylated derivatives were easy to prepare; in addition, acylation was more specific than trimethylsilylation for the reaction with hydroxyl groups in the molecule.

INTRODUCTION

As many N-nitroso compounds are potent carcinogens in experimental animals, much attention has been paid to the occurrence and possible formation of these compounds in the human environment. In recent years marked progress has been made in the trace analysis of volatile N-nitrosamines, but the lack of suitable analytical methods for less volatile or non-volatile N-nitroso compounds has hindered attempts to investigate the occurrence and formation of these compounds in the environment.

Recent studies have revealed that various less or non-volatile hydroxylated N-nitrosamines are widely distributed or formed in the human environment, *e.g.*, N-nitroso-3-hydroxypyrrolidine (NHPYR), a decarboxylated product of N-nitroso-hydroxyproline, has been reported to occur in some cured meat products after they are fried[1–5]. The presence of N-nitrosodiethanolamine (NDELA) in cutting fluids[6–8] and various cosmetics[9] has been confirmed. Some hydroxylated N-nitrosamines have also been reported to be formed by the reaction of spermidine with nitrite under

acidic conditions[10,11]. Moreover, various hydroxylated N-nitrosamines have been isolated as the *in vivo* or *in vitro* metabolites of volatile N-nitrosamines[12–14].

Several methods for the analysis of hydroxylated N-nitrosamines have been reported, including high-performance liquid chromatography (HPLC) combined with a thermal energy analyser (TEA)[6,9], gas chromatography (GC) with a TEA, and high-resolution mass spectrometry (MS) after conversion of the N-nitrosamine into a volatile derivative.

The most convenient procedure for the analysis of these non-volatile compounds appears to be HPLC–TEA but an unknown TEA-positive peak that appears in the chromatogram cannot be identified. The only reliable technique available today for the unequivocal confirmation of the presence of nitrosamines is considered to be GC–MS. For the preparation of volatile derivatives from non-volatile nitroso compounds, especially hydroxylated nitrosamines, several methods of derivatization have been proposed, including trifluoroacylation[2,5,10], trimethylsilylation[4,11,15] and methylation[3,6,16]. However, the compounds adopted in the work described above were limited in number, and only a few compounds have so far been examined. It is, therefore, not clear whether these reactions can be applied to the derivatization of various hydroxylated N-nitrosamines.

In this study, six hydroxylated N-nitrosamines were derivatized by acylation, trifluoroacylation, trimethylsilylation and methylation. The sensitivities and volatilities during GC analysis and the stabilities of these derivatives were then compared. It was found that only acylation and trimethylsilylation satisfactorily yielded volatile derivatives of the six test compounds. Methods for the preparation of the derivatives and conditions for GC analysis are described below.

EXPERIMENTAL

Reagents

All reagents were of analytical-reagent grade. NDELA[17], N-nitrosodiisopropanolamine (ND-i-PLA)[18], NHPYR[12] and N-nitroso-4-hydroxypiperidine (NHPIP)[13] were synthesized by the reaction of the corresponding amines and nitrite, and were purified by silica gel column chromatography according to published methods[12,13,18]. N-Nitroso-N-butyl-N-2-hydroxybutylamine (NBHB-2)[19,20] and N-nitroso-N-methyl-N-4-hydroxybutylamine (NMHB-4)[19,20] were kindly provided by Dr. M. Okada of the Tokyo Biochemical Research Institute. The purity of these compounds was checked by thin-layer chromatography (TLC) (Table I), GC and GC–MS.

N-Methylbistrifluoroacetamide (MBTFA) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were obtained from Pierce (Rockford, Ill., U.S.A.), and methy iodide, acetic anhydride (AA), trifluoroacetic anhydride (TFA) and other reagents from Wako Pure Chemicals (Tokyo, Japan). These reagents were employed without further purification, except for methyl iodide, which was purified by distillation.

Preparation of derivatives

Test solutions. Each test nitrosamine was dissolved in ethanol–ethyl acetate (1:4) to give a 500 μg/ml solution. For preparing a mixed nitrosamine solution, six

TABLE I

R_F VALUES OF THE HYDROXYLATED N-NITROSAMINES ON THIN-LAYER CHROMATOGRAMS

Abbreviations: NHPYR = N-nitroso-3-hydroxypyrrolidine; NHPIP = N-nitroso-4-hydroxypiperidine; NMHB-4 = N-nitroso-N-methyl-N-4-hydroxybutylamine; NBHB-2 = N-nitroso-N-butyl-N-2-hydroxybutylamine; NDELA = N-nitrosodiethanolamine; ND-i-PLA = N-nitrosodi-iso-propanolamine.

Solvent system	*NHPYR*	*NHPIP*	*NMHB-4*	*NBHB-2*	*NDELA*	*ND-i-PLA*
Diethyl ether–methanol (3:1)	0.80	0.80	0.80	1.00	0.75	0.80
Dichloromethane–ethyl acetate (4:1)	0.30	0.45	0.40	0.75	0.15	0.25
Acetonitrile–acetic acid (99:1)	0.83	0.83	0.83	0.95	0.83	0.80
Toluene–ethanol (9:1)	0.35	0.45	0.38	0.65	0.35	0.38
n-Hexane–diethyl ether–dichloromethane (4:3:2)	0.13	0.25	0.15	0.50	0.50	0.15

nitrosamines were dissolved in the same solvent to contain each nitrosamine at a concentration of 500 μg/ml. A 0.2-ml aliquot of a test solution was taken in a reaction vial (screw-capped septum vials, obtained from Pierce), followed by evaporation to dryness under a stream of nitrogen.

Acylation. A 0.2-ml volume of AA and pyridine (dried over potassium hydroxide) was added to each vial containing the test nitrosamine(s), followed by standing overnight at room temperature. Aliquots (1–5 μl) of the acylated (AC) derivatives were used for GC and GC–MS analyses.

Trifluoroacylation. Two methods were examined for the production of trifluoroacylated derivatives: (a) 0.2 ml of TFA was added to the vial followed by standing for 30 min at room temperature, the excess of TFA was evaporated under a stream of nitrogen and the residue was dissolved in 0.2 ml of dichloromethane; and (b) 0.2 ml of MBTFA was added to the vial, which was then heated at 60° for 2 h. Aliquots (1–5 μl) of the reaction mixtures, (a) or (b), were used for GC and GC–MS analyses.

Trimethylsilylation. A 0.2-ml volume of MSTFA was added to the vial, followed by standing at room temperature for 30 min. Aliquots (1–5 μl) of the reaction mixtures were analysed by GC and GC–MS.

Methylation. O-Methyl ether (ME) derivatives of test hydroxylated nitrosamines were prepared according to the method of Sen *et al.*[16].

Operating conditions for GC and GC–MS

A Shimadzu GC-5APF gas chromatograph, equipped with a flame-ionization detector (FID), was used. For the study of sensitivity and volatility of the derivatives, a glass column (3 m × 3 mm I.D.) packed with 15% SE-30 on Chromosorb W (60–80 mesh) was employed. The free forms of NDELA and ND-i-PLA were determined on a glass column (1 m × 3 mm I.D.) packed with 3% SE-30 on Chromosorb W (60–80 mesh). The operating conditions for GC were as follows: carrier gas (nitrogen) flow-rate, 40 ml/min; flame-ionization detector; hydrogen flow-rate,

20 ml/min; air flow-rate, 600 ml/min; injection port temperature, 240°; column temperature, varied from 130° to 200° depending on the derivatives examined.

A Shimadzu-LKB 9000 gas chromatograph–mass spectrometer was employed for GC–MS analysis of the derivatives of test nitrosamines. The operating conditions for GC–MS were as follows: glass column (2 m × 3 mm I.D.), packed with 3% OV-17 on Shimalite W (60–80 mesh); carrier gas (helium) flow-rate, 30 ml/min; injection port temperature, 240°; oven temperature, varied from 130° to 200° depending on the derivatives examined; separator temperature, 250°; ion-source temperature, 270°; trap current, 60 mA; electron energy, 70 eV; accelerating voltage, 3.5 kV.

RESULTS AND DISCUSSION

Each test hydroxylated N-nitrosamine was derivatized by acylation, trifluoroacylation, trimethylsilylation and methylation, and the resulting products were examined by GC–MS. All of the hydroxylated nitrosamines were derivatized by either acylation or trimethylsilylation. For trifluoroacylation, we initially used TFA in order to derivatize the nitrosamines; however, no peak corresponding to the trifluoroacylated product of NBHB-2 (TFA-NBHB-2) could be observed, probable owing to the instability of the derivative. It was also found that a substantial loss of the trifluoroacylated derivative of NHPYR (TFA-NHPYR) occurred during the evaporation of the excess of TFA, probably owing to the instability or high volatility of the product. Consequently, MBTFA was employed instead of TFA for the trifluoroacylation of NHPYR and NBHB-2. The yield of TFA-NHPYR by this method was found to be fairly high and the product was apparently stable, but no TFA-NBHB-2 could be formed with MBTFA. The ME derivatives of hydroxylated N-nitrosamines were found to be the most stable of the four derivatives tested. However, no peak corresponding to ME-NMHB-4 could be observed on the chromatogram after the derivatization of NMHB-4 with methyl iodide, and this was confirmed by GC–MS.

Table II gives the retention times and minimum detection limits for four different derivatives of six hydroxylated nitrosamines and those of the original underivatized nitrosamines. A free form of hydroxylated nitrosamine may be adsorbed on the support surface or column wall and cause a decrease in the sensitivity of GC detection; in addition, owing to an apparent broad and tailing peak, a linear correlation was not observed between the peak areas and concentrations of the test compounds. This effect was pronounced with NDELA and ND-i-PLA, which have two hydroxyl groups in their molecules. After blocking the hydroxyl groups in these compounds by the derivatization, the peaks appearing on the gas chromatogram became symmetrical with shorter retention times, and their minimum detection limits determined by GC were as low as 5–10 ng. Typical gas chromatograms obtained with AC and TMS derivatives of the six hydroxylated nitrosamines are shown in Figs. 1 and 2, respectively.

It can be seen that the peaks of these derivatives are well resolved, with symmetrical peaks that are easily distinguishable from one another. Linear relationships were observed between the peak heights or peak areas and the amounts of the derivatives in the range from 5 (10) to 100 ng. The retention times of the AC, TFA and ME derivatives of the test compounds obtained with different column packings

TABLE II

RETENTION TIMES AND MINIMUM DETECTION LIMITS OF THE FOUR DERIVATIVES OF HYDROXYLATED N-NITROSAMINES DETERMINED BY GC

Column: 15% SE-30, 3 m × 3 mm I.D.

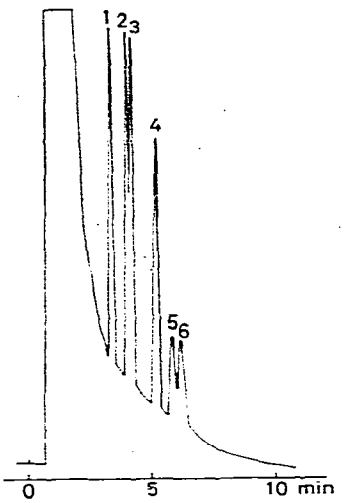
Compound	*Derivative*	*Column temp. (°C)*	*Retention time (min)*	*Minimum detection limit (ng)*
NHPYR	free form	190	3.8	2500
	Acetyl	200	3.3	5
	TFA	150	5.4	5
	TMS	170	6.6	5
	Methyl	180	4.0	5
NHPIP	free form	190	4.2	1000
	Acetyl	200	4.0	5
	TFA	150	6.6	5
	TMS	170	8.4	5
	Methyl	180	5.0	5
NMHB-4	free form	190	3.8	2000
	Acetyl	200	4.2	5
	TFA	150	7.1	5
	TMS	170	11.5	5
	Methyl	**	**	**
NBHB-2	free form	190	5.5	1000
	Acetyl	200	5.2	5
	TFA	**	**	**
	Methyl	180	6.5	5
NDELA	free form*	130	9.1	20000
	Acetyl	200	5.8	10
	TFA	150	5.7	10
	TMS	170	13.7	5
	Methyl	180	4.1	5
ND-i-PLA	free form*	130	5.2	20000
	Acetyl	200	6.2	10
	TFA	150	5.9	10
	TMS	170	14.5	10
	Methyl	180	5.2	5

* Column: 3% SE-30, 1 m × 3 mm I.D.

** No peak corresponding to the methyl derivatives of NMHB-4 and the TFA derivative of NBHB-2 appeared on the gas chromatogram.

are given in Tables III–V. The mass spectra of the AC derivatives and their probable structures are shown in Fig. 3.

Of the four derivatization reactions, acylation was found to be the most suitable; the AC derivatives were not only easy to prepare and fairly stable, but also highly volatile and sensitive to GC detection. In addition, the AC derivatives, in comparison with the other derivatives, were much more easily confirmed by GC–MS. MSTFA is known to react with carboxyl groups[21], while acylation is more specific for the reaction with hydroxyl groups in the molecule than trimethylsilylation, which is also capable of derivatizing all of the test compounds. From these findings, it can be concluded that the most suitable and convenient technique for the derivatization of hydroxylated N-nitrosamines is acylation with AA. The proposed method may be



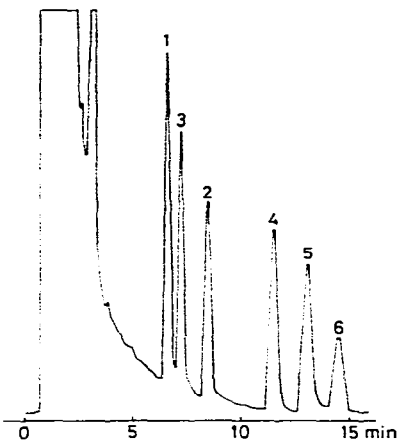


Fig. 1. Gas–liquid chromatogram of the acetyl derivatives of hydroxylated N-nitrosamines. Sample size: 100 ng of each nitrosamine. Column: 15% SE-30, 3 m × 3 mm I.D. Column temperature: 200°. Peaks: 1, N-nitroso-3-hydroxypyrrolidine; 2, N-nitroso-4-hydroxypiperidine; 3, N-nitroso-N-methyl-N-4-hydroxybutylamine; 4, N-nitroso-N-butyl-N-2-hydroxybutylamine; 5, N-nitrosodiethanolamine; 6, N-nitrosodiisopropanolamine.

Fig. 2. Gas–liquid chromatogram of the trimethylsilyl derivatives of hydroxylated N-nitrosamines. Sample size: 100 ng of each nitrosamine. Column: 15% SE-30, 3 m × 3 mm I.D. Column temperature: 170°. Peaks as in Fig. 1.

TABLE III

RETENTION TIMES OF THE O-ACETYL DERIVATIVES OF HYDROXYLATED N-NITROSAMINES OBTAINED WITH DIFFERENT COLUMN PACKINGS

Column			*Retention time (min)*					
Packing	*Length (m)*	*Temp. (°C)*	*NHPYR*	*NHPIP*	*NMHB-4*	*NBHB-2*	*NDELA*	*ND-i-PLA*
20% PEG-6000	1	200	9.8	10.2	9.4	4.9	—	—
15% DEGS	1	180	10.2	10.0	9.2	3.8	25.0	11.2
1% XE-60	1.5	170	4.4	4.7	5.2	3.4	10.5	7.1
0.5% EGA	1.5	150	4.1	4.4	4.8	4.3	12.9	8.4
3% OV-17	2	180	5.3	6.9	6.2	5.9	10.8	9.2
15% SE-30	3	200	3.3	4.0	4.2	5.2	5.8	6.2

TABLE IV

RETENTION TIMES OF THE O-TRIFLUOROACETYL DERIVATIVES OF HYDROXYLATED N-NITROSAMINES OBTAINED WITH DIFFERENT COLUMN PACKINGS

Column			*Retention time (min)*					
Packing	*Length (m)*	*Temp. (°C)*	*NHPYR*	*NHPIP*	*NMHB-4*	*NBHB-2*	*NDELA*	*ND-i-PLA*
1% XE-60	1.5	150	4.5	4.1	4.9	—*	1.0	1.8
0.5% EGA	1.5	130	4.5	4.7	5.5	—	1.0	1.8
3% OV-17	2	150	2.7	5.7	5.7	—	2.5	2.6
15% SE-30	3	150	5.4	6.6	7.1	—	5.7	5.9

* No peak corresponding to the trifluoroacetyl derivative of NBHB-2 appeared.

TABLE V

RETENTION TIMES OF THE O-METHYL ETHER DERIVATIVES OF HYDROXYLATED N-NITROSAMINES OBTAINED WITH DIFFERENT COLUMN PACKINGS

Column			*Retention time (min)*					
Packing	*Length (m)*	*Temp. (°C)*	*NHPYR*	*NHPIP*	*NMHB-4*	*NBHB-2*	*NDELA*	*ND-i-PLA*
20% PEG-6000	1	190	6.1	5.7	—*	2.9	3.9	3.3
15% DEGS	1	180	4.0	3.7	—	1.6	2.5	1.9
1% XE-60	1.5	170	2.4	2.3	—	1.8	1.7	1.6
0.5% EGA	1.5	150	2.1	1.9	—	1.6	1.6	1.6
3% OV-17	2	170	4.5	4.9	—	4.7	3.6	4.2
15% SE-30	3	180	4.0	5.0	—	6.5	4.1	5.2

* No peak corresponding to the methyl derivative of NMBH-4 appeared.

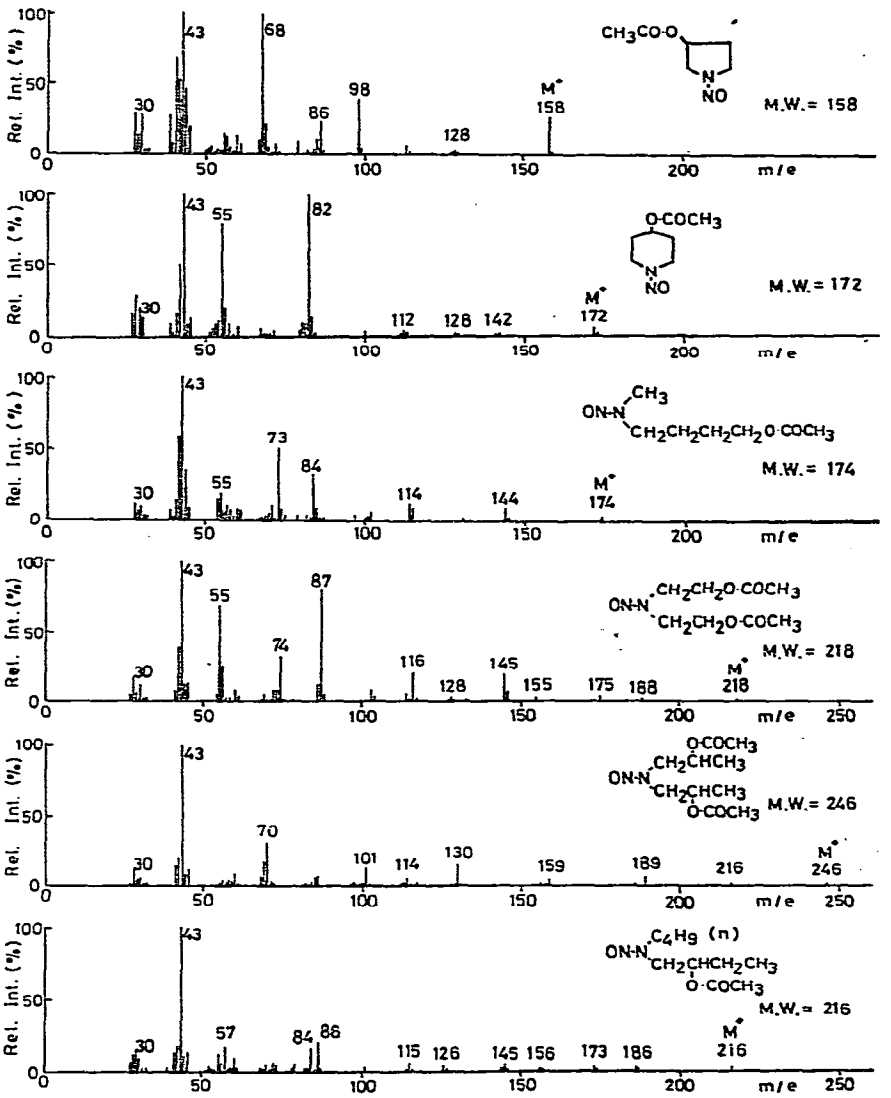


Fig. 3. Mass spectra of the acetyl derivatives of hydroxylated N-nitrosamines.

applicable to the analysis and confirmation of these hydroxylated N-nitrosamines at the parts per billion (microgram per kilogram) levels in foods. Attempts to analyse these compounds in the human environment, particularly in foods, are currently being conducted in our laboratory.

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