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N-Nitrosomorpholine and other volatile N-nitrosamines in snuff tobacco[1]

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Abstract

Ten popular snuff brands from the USA and Sweden were analyzed for volatile N-nitrosamines (VNA). Seven of these samples contained between 20 and 700 p.p.b. of N-nitrosomorpholine (NMOR), a strong animal carcinogen. Some of the snuff containers which were made of waxed cardboard contained morpholine. This observation and a model study with the container waxes plus [^{14}C]morpholine indicate that NMOR possibly can be formed by way of diffusion of the morpholine into the snuff and subsequent N-nitrosation. The VNA including NMOR (60–1150 p.p.b.) together with N-nitrosodiethanolamine (NDELA; 225–3300 p.p.b.) and the four tobacco-specific N-nitrosamines (TSNA; 1300–80 000 p.p.b.) contribute significantly to the carcinogenic potential of snuff. This tobacco product, although a known human carcinogen, is becoming increasingly popular especially among young people in the USA and Sweden. A recently introduced Swedish brand with individual snuff portions wrapped in aluminum foil was free of VNA (<2 p.p.b.) and contained relatively low levels of NDELA (290 p.p.b.) and TSNA (4200 p.p.b.). This indicates that practical approaches towards lowering N-nitrosamine levels in these snuff products are available.

Introduction

Snuff use has been suggested as a smokeless alternative to cigarettes (1–3). Although snuff dipping has not been demonstrated to represent a risk factor for lung cancer, its association with increased risks for cancer of the oral cavity and cancer of the pharynx has been established (4,5). Snuff has induced lesions of the oral mucosa in rats (6) and it has been found to contain relatively high levels of carcinogenic, tobacco-specific N-nitrosamines (TSNA*, 1–80 p.p.m.). These compounds are formed during tobacco processing and, most likely also during chewing of tobacco, as indicated by the presence of up to 0.9 μg/ml TSNA in the saliva of snuff dippers (7,8). US snuff tobaccos also contained up to 7 p.p.m. of the carcinogenic N-nitrosodiethanolamine (NDELA, 9).

Further analytical investigations revealed that snuff tobaccos also contain volatile carcinogenic N-nitrosamines (VNA) including N-nitrosomorpholine (NMOR). These studies are the subject of this communication.

Materials and Methods

Apparatus

For the analyses of the VNA in a concentrate from snuff, we employed a modified gas chromatography-thermal energy analyzer (GC-TEA) system and gas-chromatographic conditions described previously (9,10). Mass spectral analyses were performed on a Hewlett-Packard Model 5710-5980 instrument.

Snuff

Commercial US snuff products were purchased in 1981 in New York and in Tennessee; Swedish snuff originated from Stockholm. A recently introduced snuff brand (USA V) was purchased in North Carolina. All snuff samples were stored in a cold room (3°C) and were opened only immediately before the analyses.

Reagents

The reference mixture of seven VNA including NMOR was purchased from Thermo Electron Corp., Waltham, MA, USA. 2,6-Dimethylmorpholine was obtained as a *cis*- and *trans*-mixture from Aldrich Chemical Co., Inc., Milwaukee, WI, USA. Its *cis*-isomer (≅65%) was isolated and purified by preparative GC (12 mm o.d. x 4 m stainless steel column, packed with 10% UC-98 on Chromosorb WAW DMCS; column temperature 150°C). The purified *cis*-isomer was nitrosated with sodium nitrite and 0.1 N acetic acid (pH 4). The resulting N-nitroso-*cis*-2,6-dimethylmorpholine (NDMMOR) was purified by column chromatography with dichloromethane (DCM) on basic alumina (Woelm; act. II-III). The purity of NDMMOR was then ascertained by GC-TEA.

[^{14}C-U]NMOR was prepared by nitrosation of [^{14}C-U]morpholine (24.7 μCi/μM; New England Nuclear, Boston, MA, USA) with $NaNO_2$ and 0.1 N acetic acid at pH 4. The reaction mixture was extracted with DCM and purified by column chromatography on basic alumina (Woelm; act. II-III). The radiopurity of [^{14}C]NMOR was ascertained by repeated column chromatography until the specific activity was stable.

Methanol, acetone, ascorbic acid and Celite 545 were obtained from Fisher Scientific Co., Springfield, NJ. DCM was freshly redistilled over an excess of ascorbic acid assuring that the solvent was free of VNA. All other solvents and agents were free of VNA according to GC-TEA analysis.

Snuff analysis for VNA

Twenty grams of snuff were extracted for 2 h under magnetic stirring with 150 ml citrate phosphate buffer (pH 4.5) containing 20 mM ascorbic acid and [^{14}C]NMOR (63 000 d.p.m. = 0.143 μg) as an internal standard. The mixture was filtered through washed Celite 545 and the filtrate was extracted 4 times with 150 ml DCM. The combined DCM-layers were dried (Na_2SO_4) and reduced to ~5 ml. The VNA concentrate was chromatographed on a column of 65 g basic alumina (Woelm; act. II-III) with DCM. The resulting 50-ml fractions were monitored for β-activity. In most cases, the VNA eluted in fractions 2–4. These fractions were concentrated to 1 ml, and aliquots were counted for determining the recovery rate; other aliquots were analyzed by GC-TEA to determine the VNA (10).

In two cases we used GC-mass spectrometry (MS) in order to confirm the identity of the compound which had the retention time of NMOR in the GC-TEA system. These analyses were started with 50 g snuff. The VNA fraction was further enriched by a second chromatographic step using 650 g of silica gel prior to GC-MS analyses.

In order to determine whether artifacts led to formation of NMOR (and of other VNA) during the extraction and analysis, we added 31.2 mg and 3.12 mg of *cis*-2,6-dimethylmorpholine to the snuff in two separate analyses. This agent was recommended by Mirvish *et al.* for monitoring artifact formation (11). The average retention time for NMOR in the GC-TEA system was 14 min, while that of NDMMOR was 11 min, thus allowing distinction.

NMOR analysis of packaging materials

About 10 g of container material (brown cardboard or colored plastic) were

[1]This publication is dedicated to the founder of the American Health Foundation, Dr. Ernst L. Wynder, on the occasion of the 10th anniversary of the Naylor Dana Institute for Disease Prevention.

*Abbreviations: TSNA, tobacco specific N-nitrosamines; NDELA, N-nitrosodiethanolamine; VNA, volatile carcinogenic N-nitrosamines; NMOR, N-nitrosomorpholine; GC, gas chromatography; TEA, thermal energy analyzer; NDMMOR, N-nitroso-*cis*-2,6-dimethylmorpholine; DCM, dichloromethane; MS, mass spectrometry; NDMA, N-nitrosodimethylamine; NNN, N′-nitrosonornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAT, N′-nitrosoanatabine; NAB, N′-nitrosoanabasine.

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homogenized in a blender, then added to 200 ml of citrate buffer (pH 4.5) containing 0.7 g ascorbic acid and [^{14}C]NMOR as an internal standard. After 2 h of mixing, the homogenate was filtered and extracted four times with 200 ml DCM. The analyses of container wax extracts for NMOR (and other VNA) were continued in the same manner as those of the snuff samples.

Determination of morpholine

Twenty-five grams of snuff were suspended in 200 ml water containing [^{14}C]morpholine (173 000 d.p.m. = 0.30 μg) and were extracted overnight under magnetic stirring. The extract was filtered through Celite, washed with water and acidified with 18 N acetic acid to pH 4.5. The acidic solution was extracted three times with 200 ml ether. Two grams of $NaNO_2$ were then added to the extracted aqueous layer. After 2 h of magnetic stirring the aqueous solution was extracted three times with DCM. The combined DCM extracts were dried (Na_2SO_4), concentrated to ~2 ml, and subsequently analyzed for NMOR as described above.

The morpholine analyses of the containers required ~10 g of ground materials. The procedure was the same as that for snuff.

Model study for transfer of morpholine

Twenty cardboard snuff containers USA III (≅170 g) were extracted in a soxhlet with 3000 ml n-hexane. The extract was dried (Na_2SO_4) and reduced to ~300 ml. The extracted material (≅40 g) had a waxy consistency. Four aliquots were placed into Petri dishes (internal diameter 85 mm) together with 0.5 ml each of a solution of [^{14}C]morpholine in ethanol (1516 000 d.p.m. = 2.6 μg). After the solvent had evaporated, ~17 g of snuff were placed on top of the wax layer of each Petri dish. The dishes were covered and stored in the dark. The snuff used for this test was free of NMOR. The Petri dishes were left at ambient temperatures (≅20°C) for one month; then the snuff was removed and analyzed for [^{14}C]NMOR.

Results

Figure 1 presents a GC-TEA trace of the VNA concentrate from one of the US snuff samples. Significantly, this chromatogram is from one of seven VNA concentrates among the analyzed snuff samples which had a major peak with the retention time of NMOR. Two analyses and extensive clean-up of 50 g snuff samples each were carried out to enable us to identify NMOR in these VNA concentrates by GC-MS (Figure 2). Table I lists the results for VNA and morpholine in the 10 snuff samples including the NMOR and morpholine data for the snuff containers. The detection limit for individual VNA varied between 0.5 and 1 p.p.b. for snuff samples of 20 g. According to repeated analyses the standard deviation for NMOR was less than ±10%. In the presence of relatively high concentrations of NMOR (>100 p.p.b.) standard deviations were within ±7%. The recovery rate of NMOR was between 50 and 70% as measured with [^{14}C]NMOR as an internal standard. NMOR was the major nitrosamine in the snuff containers but, in a few cases, traces of NDMA and NPYR (<3 p.p.b.) were also found. As described under **Materials and Methods**, the morpholine analyses were carried out with the N-nitrosation method and with [^{14}C]morpholine as an internal standard. The detection limit was 2 p.p.b. and the recovery rates varied between 70 and 80%. Since we found morpholine in snuff as well as in the containers, we investigated the possibility of artifactual formation of NMOR during extraction and analysis (11). When 31.2 mg and 3.12 mg of *cis*-2,6-dimethylmorpholine were added to the snuff, 1.62 μg and 0.21 μg of NDMMOR, respectively, were found. This demonstrates that 0.0052% and 0.0067% of the added amine have been nitrosated. This low degree of N-nitrosation indicates that the NMOR values observed in this study are not significantly increased by artifacts (<0.4 p.p.b.). Table I shows that the concentration of morpholine in the snuff and snuff containers is not the yield determining factor for NMOR. Based on past experiences, we know that the processing of snuff tobacco and its aging affect the N-nitrosamine yield (8). Table I indicates also that the containers are a possible source of morpholine in the snuff. In order to verify this concept, the waxy extracts from the snuff containers, [^{14}C]morpholine and moist snuff which was free of NMOR (brand II, Sweden), were incubated together at room temperature in sealed Petri dishes for one month (see **Materials and Methods**). Analysis of the snuff for [^{14}C]NMOR showed in 2 parallel experiments that 0.60% and 0.51% of the [^{14}C]morpholine had diffused from the waxy layer in the bottom of the dish into the snuff and had been nitrosated to [^{14}C]NMOR. In the absence of [^{14}C]morpholine, however, the incubation of the waxy materials with snuff did not yield significant amounts of NMOR.

Table II presents an overview on VNA including NMOR, NDELA (9) and the four major tobacco-specific N-nitrosamines in snuff (7,8). For the analyses of NDELA and TSNA, we applied previously published methods (9,15). NDELA derives from the sucker growth inhibitor maleic hydrazide diethanolamine residue on tobacco (9).

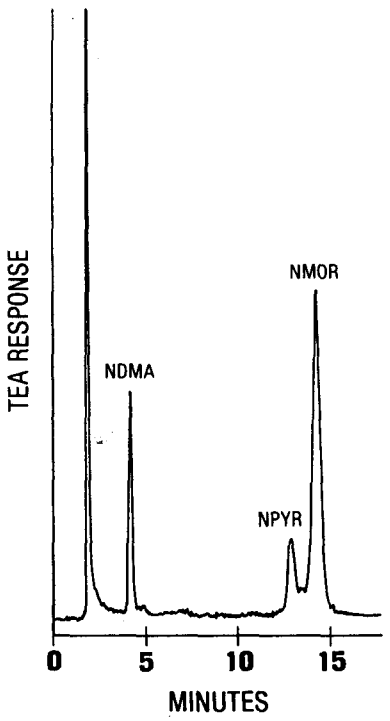

Fig. 1. Gas chromatogram of VNA concentrate from snuff-USA IV.

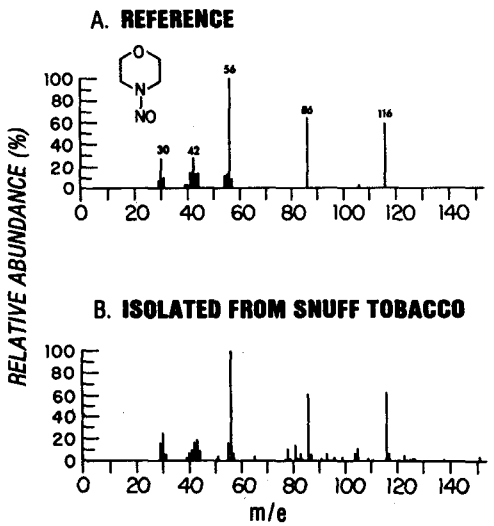

Fig. 2. Mass spectra of NMOR reference and isolate from snuff-USA II.

Discussion

A few years ago we detected traces of VNA in snuff in a preliminary investigation (10). This study confirms that 5 popular US snuff brands and 5 Swedish snuff products contain N-nitrosamines. Seven out of 10 snuff brands contained NMOR (10–690 p.p.b.). Only one report in the literature refers to morpholine, the precursor for NMOR, as being detected in tobacco samples (12). Since we found appreciable amounts of morpholine (90–4830 p.p.b.) in the waxed cardboard containers which are used to package the snuff, we hypothesized that this compound may have partially diffused from the containers into the snuff where it may have contributed to morpholine and NMOR. This is of significance because NMOR is a strong animal carcinogen and morpholine is known to be N-nitrosatable *in vivo* (13). The diffusion of morpholine into the snuff was then demonstrated in a model experiment with [^{14}C]morpholine. However, the finding of 19 400 p.p.b. of morpholine in a sample of loose leaf chewing tobacco, which was packaged in an aluminum pouch, indicates that there are other conceivable sources of morpholine contamination in processed tobacco, such as "casing solutions" (14). The latter are mixtures of hygroscopic agents and volatile and nonvolatile flavoring components which are applied to snuff. The exact composition of casing solutions are trade secrets.

The data in Table II should be evaluated in light of the findings from bioassays in Syrian golden hamsters. These have documented that NDMA is a very strong carcinogen, NMOR and NNK are strong carcinogens of about equal potency, NPYR, NNN and NDELA are moderately active carcinogens and NAT and NAB are weakly active (13,15–16). The carcinogenic potential of snuff must be kept at a minimum because snuff products are proven human carcinogens (3,4,17). Processing methods and packaging materials for snuff should be carefully selected so as to preclude the presence of morpholine which can contribute to the formation of NMOR in snuff as well as *in vivo* (13).

As Table II demonstrates, it is possible to offer snuff which is significantly lower in N-nitrosamines (Sample V USA and Sample V Sweden). N-Nitrosamines are the only known genotoxic agents in this human carcinogen. Their reduction in, or practical elimination from, snuff products is necessary as a measure of risk reduction.

Table I. Nitrosomorpholine and morpholine in snuff and snuff containers (p.p.b.)[a]

Snuff brand		Snuff tobacco		Snuff container	
		NMOR	Morpholine	NMOR	Morpholine
USA	I	24	2800	34	845
	II	690	1500	10	170
	III	690	4000	230	4740
	IV	630	3200	4	90
	V	31	2200	3	140
Sweden	I	44	820	4	1750
	II	(–)	200	3	460
	III	(–)	780	13	4830
	IV	10	940	23	4290
	V	(–)	2500	N.D.	N.D.

[a]Based on dry weight in case of snuff; uncorrected for moisture in case of snuff containers. The latter had contained snuff previously. Containers of USA I–III and Sweden I–IV were cardboard boxes with a metal lid, USA IV plastic containers with individual snuff portions in porous paper bags; USA V plastic container; Sweden V individual snuff portions in Al-bags. (–), below detection limit (<2 p.p.b.). N.D. = not determined.

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Table II. N-Nitrosamines in snuff (p.p.b.)[a,b]

Snuff brand		Volatile N-nitrosamines				Tobacco-specific N-nitrosamines			
		NDMA	NPYR	NMOR	NDELA	NNN[c]	NNK[c]	NAT[c]	NAB[c]
USA	I	215	(–)	24	760	2200	600	1700	100
	II	37	120	690	1700	19 000	2400	19 000	800
	III	100	360	690	3300	33 000	4600	40 000	1900
	IV	92	110	630	290	20 000	8300	9100	500
	V	(–)	(–)	31	600	830	210	240	10
Sweden	I	22	(–)	44	240	5700	1700	900	140
	II	60	(–)	(–)	225	6100	1000	2200	80
	III	14	210	(–)	390	5300	1400	2400	70
	IV	30	50	10	310	4000	610	1400	80
	V	(–)	(–)	(–)	290	2000	800	1400	40

[a]Values are based on dry weight. (–), below detection limit (< 2 p.p.b.). [b]Values for nitrosodiethylamine in snuff were below detection limit (< 2 p.p.b.) except Sweden I, II, III and IV which had values of 6, 4, 12 and 5 p.p.b., respectively. [c]NNN, N'-nitrosonornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAT, N'-nitrosoanatabine; NAB, N'-nitrosoanabasine.

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