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Tobacco-specific *N*-nitrosamines in the saliva of habitual male snuff dippers

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Saliva was collected every ten minutes from habitual male snuff dippers and analysed for tobacco-specific *N*-nitrosamines (TSNA). Detectable levels of at least two TSNA were found in all samples collected between 10 and 30 minutes after the snuff had been placed in the mouth. Total concentrations of TSNA up to 241 ng/g were found in the saliva. Trace levels of TSNA were still found in the saliva 20 minutes after the snuff had been removed.

Keywords: snuff, N-nitrosamines, saliva, tobacco

Introduction

Retrospective studies have indicated a connection between snuff dipping and oral cancer (Axéll *et al.* 1978, Winn *et al.* 1981). Recently, the International Agency for Research on Cancer (IARC) has stated that "there is sufficient evidence that oral use of snuffs of the types commonly used in North America and Western Europe is carcinogenic to humans"(IARC 1985). Tobacco-specific *N*-nitrosamines (TSNA) are the most abundant carcinogens identified in tobacco and tobacco smoke, and are formed during the ageing, curing and fermentation of tobacco. We have previously reported on the levels of carcinogenic volatile and TSNA in snuff and chewing tobacco on the Swedish market (Österdahl and Slorach 1983, 1984).

TSNA have recently been found in the saliva of snuff-dipping women in the USA (Hoffmann and Adams 1981). Both volatile and TSNA have also been detected in the saliva of habitual chewers of tobacco with lime or betel quid, and in the saliva of masheri users in India (Sipahimalani *et al.* 1984. Nair *et al.* 1985, Bhide *et al.* 1986).

In Sweden the use of snuff has increased considerably during the last two decades and the total sale is now over 4500 tonnes per year. About 13% of adult men use snuff daily in Sweden, and in 1983 about 29% of the 16 year-old schoolboys were snuff dippers.

The purpose of the present study was to follow up the previous work on TSNA by determining the levels of these substances in the saliva of snuff dippers.

Experimental

Chemicals

N'-Nitrosonornicotine (NNN), *N'*-nitrosoanatabine (NAT) and 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were gifts from Dr J. D. Adams, Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, NY, USA. Standard NNN solution (100 μg/ml) in chloroform was

obtained from the Thermo Electron Corp. (Waltham, MA, USA). Dichloromethane (analytical grade) was obtained from Riedel de Haen AG (Seelze-Hannover, FRG), and 2,2,4-trimethylpentane (analytical grade) was purchased from E. Merck AG (Darmstadt, FRG). Extrelut (E. Merck AG) was dried overnight at about 150°C prior to use and stored at the same temperature. Sodium hydroxide (reagent grade), deionized water, and the organic solvents used were checked to ensure the absence of interfering substances.

Snuff

Two different types of Swedish moist snuff were used, one sold in 50 g boxes (snuff dipper no. 1), and the other packed as individual snuff portions of about 2 g in porous paper sachets (snuff dippers nos. 2–4).

Collection of saliva

Staff of the Swedish National Food Administration were donors of saliva. All donors were habitual male snuff dippers, aged 22–41 years. The saliva was obtained just before, during and after snuff dipping. About 2 ml saliva was collected in test-tubes at each time and analysed within 5 min.

Methods

Saliva samples were weighed and mixed with 1 ml of 1 M sodium hydroxide. The mixture was placed on an Extrelut column (7 cm long, 2 cm ID), and the sample test-tube was rinsed with 2 × 1 ml of water, which was also placed on the column. After 15 min, the column was eluted with dichloromethane (4 × 25 ml), and the eluate was concentrated to about 0·3 ml in a water-bath at 55°C. Towards the end of the concentration process, 200 µl of 2,2,4-trimethylpentane was added as a 'keeper'. The final volume was measured with a 1000 µl Hamilton syringe.

Analyses were carried out using an isothermal gas-liquid chromatograph (GLC, Varian 2700, Palo Alto, CA, USA) interfaced with a Thermal Energy Analyser (TEA, Model 502, Thermo Electron Corp.). The furnace was removed from the TEA and connected to the GLC column via a 5·5 cm long glass tube. For detection and quantification, 7·0 µl portions were analysed against external standards by injection into a 1·8 m × 1·9 mm ID glass column containing 10% UCW-982 on Chromosorb W, AW-DMCS, 80/100 mesh. The GLC-TEA conditions were: column temperature 200°C, injector temperature 240°C, helium carrier-gas flow about 28 ml/min, furnace temperature 475°C, oxygen flow 10 ml/min, vacuum pressure about 0·5 mm Hg. A CTR Gas Stream Filter (Thermo Electron Corp.) was used.

The detection limits of the method were about 2–3 ng TSNA/g saliva.

The TSNA content of snuff was determined by a method we have previously reported (Österdahl and Slorach 1984).

Results and discussion

The possibility of formation of TSNA during the analytical procedure was reduced to a minimum by addition of sodium hydroxide and a rapid dichloromethane extraction on Extrelut.

Each set of saliva samples analysed contained saliva spiked with TSNA to determine recovery. Table 1 shows the recoveries of NNN, NAT, and NNK from these spiked saliva samples. The average recovery of the TSNA varied between 79

Table 1. Extraction yields of NNN, NAT, and NNK from saliva.

Tobacco-specific *N*-nitrosamine	Concentration (ng/ml)	No. of determinations	Recovery (%, mean ± S.D.)
NNN	30–180	14	79 ± 14
NAT	40–230	7	79 ± 9·5
NNK	35–140	15	84 ± 9·8

and 84%. No TSNA could be detected in saliva from persons who were not snuff users.

Saliva was collected from four habitual snuff dippers. The levels of TSNA in saliva during and after snuff dipping are shown in Table 2, and are not corrected for recovery. About half of the TSNA values in Table 2 are averages of two determinations of the same saliva sample. The duplicate values were within ± 6·5% of their mean. Snuff dipper no. 1 used his own snuff (with unknown TSNA content); the others used pre packed portions of snuff from the same batch, with a known content of TSNA.

No TNSA could be detected in pre-snuff dipping samples, except on one occasion in one snuff dipper (no. 4), where traces of TSNA were found. This was presumably because too short a time had elapsed since the previous snuff dipping. Samples of saliva were collected after 10, 20 and 30 min snuff dipping. In one saliva sample collected after 5 min snuff dipping detectable levels of all three TSNA were found (not shown in Table 2).

Table 2 shows that there was an appreciable variation in the level of TSNA in the saliva, not only between individual snuff dippers, but also in saliva from the same snuff dipper collected on two different days. All samples collected during the snuff dipping contained detectable levels of NNN and NAT, ranging from 3 to 140 ng/g and from trace levels to 85 ng/g, respectively. NNK was found in about 70% of the saliva samples at levels up to 16 ng/g.

The concentrations of TSNA found in the present study are of the same magnitude as those reported in the saliva of snuff dipping women, tobacco chewers and masheri users (Hoffmann and Adams 1981, Sipahimalani *et al.* 1984, Nair *et al*, 1985, Bhide *et al.* 1986). However, in our study, the levels of TSNA found in saliva were lower than those reported earlier in the saliva of snuff-dipping women, probably because the levels of TSNA in the snuff used in the present study were lower (Hoffman and Adams 1981). Variation in TSNA content in saliva collected on two different days has been observed earlier with snuff-dipping women and men chewing tobacco with lime (Hoffmann and Adams 1981, Bhide *et al.* 1986).

All the snuff dippers in the present study used the snuff for 30 min. Table 2 shows that detectable levels of TSNA could be found in saliva also after the snuff dipping period. NNN and NAT were present in all saliva samples tested 10 min after the snuff had been taken out, at levels ranging from 2 to 13 ng/g. Traces of NNN and NAT were also found in the saliva 20 min after the snuff dipping period. NNK was only detected in one saliva sample collected after the snuff had been taken out.

The snuff used by three of the subjects (nos. 2–4) was analysed before and after snuff dipping. Table 3 shows the changes in the levels of TSNA in the snuff after use. The concentrations in the snuff have been corrected for the increased weight of the snuff sachet after snuff dipping. The NAT concentration was nearly the same before

Table 2. Levels of tobacco-specific *N*-nitrosamines (TSNA) in the saliva of habitual snuff users collected during and after the snuff dipping.

Snuff dipper		Tobacco-specific *N*-nitrosamine	Day of snuff dipping	TSNA level (ng/g) in saliva at different times (min) of intake				after snuff[1]
No.	Age (yr)			10	20	30	40[2]	50[2]
1	38	NNN	1	140	55	37	–[3]	–
		NAT		85	24	17	–	–
		NNK		16	Tr[4]	ND[5]	–	–
		NNN	1[6]	77	43	41	–	–
		NAT		34	22	22	–	–
		NNK		7	Tr	ND	–	–
2	22	NNN	1	52	65	74	–	–
		NAT		21	37	41	–	–
		NNK		Tr	9	10	–	–
		NNN	2	19	62	52	13	Tr
		NAT		4	19	19	6	Tr
		NNK		ND	Tr	TR	ND	ND
3	33	NNN	1	24	12	11	–	–
		NAT		12	5	Tr	–	–
		NNK		Tr	Tr	ND	–	–
		NNN	2	3	11	16	8	Tr
		NAT		Tr	5	10	7	Tr
		NNK		ND	ND	Tr	ND	ND
4	41	NNN	1	20	38	22	3	Tr
		NAT		11	29	14	2	Tr
		NNK		ND	9	3	ND	ND
		NNN	2	6	6	15	11	–
		NAT		11	7	8	6	–
		NNK		13	3	5	Tr	–

NNN = *N'*-Nitrosonornicotine
NAT = *N'*-Nitrosoanatabine
NNK = 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone
[1] Not corrected for recovery
[2] The snuff was taken out after 30 min
[3] Not analysed
[4] Trace
[5] Not detected
[6] About 2 hrs after the previous snuff dipping

and after use, while NNN and NNK levels were lower in the used snuff of two of the three snuff dippers. The differences are small, but it is interesting to note that the levels of TSNA in the snuff after use were highest with the snuff dipper with the highest levels of TSNA in the saliva (no. 2). The total amount of TSNA in this used snuff was nearly the same as before snuff dipping, in spite of the fact that high levels of TSNA were found in the saliva. This could well be due to an *in vivo* formation of TSNA in the saliva. It has previously been reported that the NNN content of fine-cut chewing tobacco incubated with human saliva for 3 h at 37°C increased by 44% (Hecht *et al.* 1975).

The tobacco used to prepare snuff often contains very high amounts of nitrate. Bacteria can reduce nitrite to nitrate (Ralt and Tannenbaum 1981), and catalyse the nitrosation of amines (Hawksworth and Hill 1971). Thus, the differences in the levels of TSNA in the saliva might partly be explained by variations in the bacterial content in saliva. Hoffman and Adams (1981) put forward other possible explana-

Table 3. Tobacco-specific *N*-nitrosamines (TSNA) in snuff before and after snuff dipping

	Snuff dipper no.	Weight of snuff sachet, (g)		Tobacco-specific *N*-nitrosamine content (mg/kg)[1]			
		before	after	NNN	NAT	NNK	Total
Before snuff dipping				4·4[2]	3·5[2]	1·3[2]	9·2[2]
After snuff dipping	2	2·0	2·0	4·8	3·6	1·1	9·5
	3	2·1	2·3	3·6	3·8	0·9	8·3
	4	2·2	2·4	4·0	4·0	0·9	8·9

[1] Corrected for the increased weight after use
[2] Mean of two determinations

tions, namely differences in the intensities of snuff dipping and variations in rates of salivation.

From the data in Table 2 it can be calculated that the average total TSNA levels in the saliva of the four snuff dippers during 30 min snuff dipping were between 15 and 125 ng/g. According to Hoffmann and Adams (1981), an adult produces about 60 ml of saliva per h. This implies that the snuff dippers in our study were exposed to 0·9 to 7·5 μg TSNA per hr snuff dipping. It is not unusual for snuff dippers to use snuff for up to 15 h per day. Thus, they can be exposed to over 110 μg TSNA daily. Habitual snuff dippers often use 4 to 6 g of snuff, i.e. more than twice the amount used in this study by the snuff dippers nos. 2–4, and thus the exposure can be even higher. The amount of TSNA that snuff dippers may be exposed to (110 μg) can be compared with the estimated daily intake of volatile *N*-nitrosamines from food in Sweden of 0·29 μg/person (Österdahl 1988).

The saliva of snuff dippers contains considerable amounts of TSNA, which are all known to be potent carcinogens in experimental animals (Hoffmann and Hecht 1985). During snuff dipping the oral cavity of the snuff users is constantly exposed to high concentrations of these carcinogenic TSNA. The association between oral cancer and snuff dipping could well be due to the TSNA present in the saliva of snuff dippers. Furthermore, it is quite possible that exposure to TSNA may give rise to cancer at sites other than the oral cavity.

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