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# Oral mucosal changes and nicotine disposition in users of Swedish smokeless tobacco products: a comparative study

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The purpose of this study was to investigate the uptake and metabolism of nicotine by smokeless (oral) tobacco users and to find out if the less pronounced clinical changes in the oral mucosa in users of portion-bag packed oral moist snuff (*snus*) compared with the changes in the mucosa of loose *snus* users are correlated to exposure and uptake of tobacco constituents. 54 habitual users of smokeless tobacco were selected for the study: 22 loose *snus* users, 23 users of portion-bag packed *snus* and 9 users of chewing tobacco. In accordance with previous findings, less pronounced clinical changes in the oral mucosa were recorded in portion-bag users compared with loose *snus* users. The clinical findings observed in the oral mucosa of users of chewing tobacco were leukoedema and slight clinical "*snus* changes". The average intake of nicotine (measured as nicotine equivalents excreted during 24 h) for *snus* users was 35 mg, and was 50% higher for users of chewing tobacco. The average steady-state saliva cotinine concentration was about 300 ng/ml for both categories of *snus* users, which is similar to that found in smokers, while the average concentration found in users of chewing tobacco was 50% higher. There was a good correlation between saliva cotinine concentration and the 24 h intake of nicotine. The average excretion profile of nicotine was similar in all three groups of smokeless tobacco users, being on average: nicotine 8%, nicotine-GlcA 3%, cotinine 8%, cotinine-GlcA 9%, 3′-hydroxycotinine 42%, 3′hydroxycotinine-GlcA 19%, nicotine-N′-oxide 9% and cotinine-N-oxide 3%. The clinical severity of buccal mucosal changes correlated neither with the markers for exposure (i.e. nicotine and tobacco specific nitrosamines extracted from the tobacco) nor with the biological markers for uptake of tobacco constituents (i.e. nicotine equivalents excreted during 24 h and saliva cotinine concentrations).

Key words: nicotine metabolism; TSNA; oral manifestations; smokeless tobacco

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In Sweden, the use of oral moist snuff, *snus*, was already a widespread habit in the 19th century. In 1990, about 9% of the population over 15 years of age were users of moist *snus* (16% of men and 1.5% of women) and 26% were cigarette smokers. Two types of moist *snus*, loose *snus* and portion-bag packed *snus*, are produced in Sweden and these command almost 100% of the market share. Portion-bag packed *snus* was introduced about 20 years ago and has steadily gained in popularity. Of Swedish *snus* users, 73% consume only loose *snus*, 13% only portion-bag packed *snus* and 14% have a mixed habit. The average consumption of loose *snus* is 15 g/day, and of portion-bag *snus* 9 g/day (1).

The annual sales figures for chewing tobacco in Sweden are low compared with the figures for moist *snus*, 13 tons versus 5000 tons, as less than 0.002% of Swedes are users of chewing tobacco (2).

A previous study on 252 users of Swedish *snus* (3) has shown that soft tissue changes of the oral mucosa and the gingival margin are less pronounced among those who use portion-bag *snus* than among those who use loose *snus*. Further, the daily exposure to *snus* has a larger impact in the clinically recorded changes than does the number of years with regular habit. These clinical findings were supported by histological analyses.

In a study on 20 Swedish chewing tobacco users, oral mucosal changes were considered slight since the most common clinical findings were leukoedema-like changes of the buccal mucosa. In clinical and epidemiological

studies on the effects of tobacco smoke, environmental tobacco smoke and smokeless tobacco, it is of great importance to quantify exposure. Since nicotine is virtually unique to tobacco and also one of the most ubiquitous compounds, it has been widely used as a marker for exposure to tobacco and tobacco smoke and as a biological marker in body fluids for the uptake of tobacco constituents.

Recent studies on the metabolism of nicotine have shown that about 95% of the nicotine dose could be estimated by measuring nicotine and its seven main metabolites excreted in urine. At steady-state, the rate of excretion of metabolites reflects the generation rate. The daily intake of nicotine by tobacco users could thus be estimated by measuring nicotine and its metabolites in 24-h urine samples. Several studies have been performed on the uptake of nicotine by smokers (4–10), but few investigations have been carried out on oral snuff users (11–15).

The aim of this study was to investigate the uptake and metabolism of nicotine by smokeless tobacco users. Further, it was to find out if the less pronounced clinical oral mucosal changes in portion-bag *snus* users, as compared to those in loose *snus* users, are correlated to the exposure and uptake of tobacco constituents.

## Material and methods

### Subjects

The subjects selected for this study were 54 habitual users of smokeless tobacco. Loose *snus* was used by 22 subjects of mean age 38.8 ± 13.8 yr, portion-bag *snus* by 23 subjects of mean age 40.8 ± 8.7 yr, and chewing tobacco by 9 subjects of mean age 50.4 ± 9.6 yr.

The *snus* users who participated in this study were selected from a previous study comprising 252 healthy men with a regular *snus* habit for at least the previous 3 months and with no other current tobacco use. For a detailed description of the recruitment and examination procedures of the original material see ANDERSSON (3), in which loose *snus* was used by 184 subjects of mean age 36.0 ± 11.6 yr and portion-bag *snus* by 68 individuals of mean age 36.9 ± 9.9 yr.

Recruitment for the present study was based on equal daily consumption and usage of the same tobacco brand. In the original examination, 25 loose *snus* and 25 portion-bag users fulfilled these criteria. Three years later, when the material for this study was collected, 3 loose *snus* users and 2 portion-bag users had stopped using *snus*, giving a recruitment of 45 *snus* users. Of these subjects, 14 portion-bag users and 13 loose *snus* users had either changed their daily consumption pattern or the tobacco product. Eighteen *snus* users, 9 in each group, had unchanged habits, which allowed 9 matched pairs to be specifically compared.

The users of chewing tobacco were selected from another study, which comprised 20 healthy men with a mean age of 51.9 ± 12.3 yr, range 25–80 yr (16). Of these, 10 users of chewing tobacco with no other tobacco habit and who were living in the area where the present study was performed were recruited for this study. Nine of those were eventually included as one had ceased his habit.

The participants, who were all healthy volunteers, were given written information on the purpose of the study before giving their written consent to participate. The study was reviewed and approved by the institutional ethics committee at Lund University.

### Clinical examination

All subjects were called to a dental clinic and examined according to a standardized program. Questions were asked on type and brand of smokeless tobacco, years of usage, daily consumption (hr/day and g/day) and whether they placed the pinch of smokeless tobacco at one or more sites, between the gum and the buccal mucosa or the gum and the upper lip, or if they chewed the tobacco. Questions were also asked about general health, medication, previous tobacco habits and alcohol consumption.

A thorough clinical examination was carried out and lesions in the oral mucosa were recorded. Changes at the site(s) where the tobacco was regularly placed, *snus* dipper's lesion, were registered according to a four-point scale suggested by AXELL *et al.* (17). This scale is based on clinical criteria including wrinkling, thickening and colour changes of the oral mucosa. Leukoedema, defined as a greyish-white, velvet-like diffuse oedematous film covering smooth buccal mucosa, was also recorded.

### Sample collection

The participants were using their ordinary brand of smokeless tobacco *ad libitum* and recorded their daily consumption (g/day and hr/day) for seven days. On Day 6, urine samples were collected for 24 h. The volume and pH of the urine voids were then measured and 10 ml aliquots were transferred into glass tubes. On the same day, the smokeless tobacco users collected all used portions of smokeless tobacco in glass jars. On Day 7, one saliva sample (1 ml) was gathered in the following way: 30 min after the intake of a pinch of *snus* or a piece of chewing tobacco, the tobacco was spat out, and after another 30 min the mouth was rinsed with water and a whole mixed saliva sample (2 ml) was collected direct into a glass tube. The saliva and urine samples and the used samples of smokeless tobacco were kept frozen (−18 °C) until analyzed.

### Analysis of chemical constituents of smokeless tobacco products

*Nicotine* – One g of moist *snus* was suspended in 500 ml of water and sonicated for 75 min. The slurry (200 µl) was transferred to a septum vial and an appropriate amount of the internal standard, N′-methylanabasine in 800 µl of ethanol, was added. The mixture was shaken and then centrifuged at 6000 g for 10 min. Quantitative analysis was performed by gas chromatography as described below.

*Tobacco-specific nitrosamines (TSNA)* – The amount of each of four tobacco-specific nitrosamines, N′-nitrosonornicotine (NNN), N′-nitrosoanatabine (NAT), N′-nitrosoanabasine (NAB) and 4-(N′-methyl-N′-nitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), was determined using a modified method developed by SPIEGELHALDER *et al.* (18). The analytical procedure was briefly: to 1 g of moist *snus* was added a NaOH-solution (20 ml; 0.01 M; 100 ppm sodium ethylmercurythiosalicylate) and the internal standard, N-nitrosopentylpicolylamine. After adjustment to pH 8.0, the mixture was sonicated for 30 min. The slurry was then transferred to an Extrelut column and the nitrosamines were eluted with $CH_2Cl_2$, (3 × 25 ml). After evaporation of the solvent, quantitative analysis was performed by high resolution gas chromatography in combination with chemiluminescence detection.

### Analysis of nicotine and metabolites

Urine samples from the smokeless tobacco users were divided into five portions. Portion one was analyzed for ni-

cotine and cotinine, portion two for glucuronic acid conjugates of nicotine and cotinine, portion three for *trans*-3′-hydroxycotinine, portion four for glucuronic acid conjugate of *trans*-3′-hydroxycotinine, and portion five for nicotine-N′-oxide and cotinine-N-oxide. Saliva samples were analyzed for the concentrations of cotinine.

*Nicotine and cotinine* - The concentrations of cotinine in saliva and of nicotine and cotinine in urine were determined by the method of CURVALL *et al.* (19). To samples of urine (0.5 ml) were added appropriate amounts of the internal standards, N′-methylanabasine for nicotine and N′-ethylnorcotinine for cotinine, and to samples of saliva were added the internal standard for cotinine. The samples were extracted with dichloromethane (1 ml) under basic conditions (0.5 ml, 5 M NaOH) and the organic layer was separated and evaporated. Quantitative analysis was performed on a gas chromatograph (Varian model 3700, Walnut Creek, California), equipped with an all-glass capillary injector, a fused silica capillary column (SP-1000, Orion Analytica, Espo, Finland), and a thermionic specific detector (TSD, Varian). The plotting of the chromatograms and integration of the peak heights and peak areas were carried out by a Hewlett-Packard Model 3388 A (Hewlett-Packard Company, Avondale, Pa.) or a Shimatzu Model C-R3A (Tokyo, Japan) plotter/integrator. The limit of determination was 1 ng of nicotine and cotinine per ml of urine, with the precision of 10% at concentrations of 5 ng/ml.

*Nicotine and cotinine glucuronides* - The total amounts of nicotine and cotinine (i.e. both free and conjugated alkaloids) were determined after enzymatic hydrolysis of urine samples. To 0.5 ml of urine, which was diluted with an equal amount of sodium acetate buffer (0.05 M, pH 4.7), was added β-glucuronidase from *Helix pomatia* (6000 U, EC 3.2.1.31, Type HP-2, Sigma Chemical Co, USA) dissolved in sodium acetate buffer (0.2 ml). After incubation at 37°C for 4 h, the reaction mixture was analyzed for nicotine and cotinine using the same analytical procedure as described above. The concentrations of glucuronic acid conjugates of nicotine and cotinine were calculated from these data (20).

*Trans-3′-hydroxycotinine* - To samples of urine (0.5 ml), the internal standard, 3′-hydroxy-N-propylnorcotinine (4 μg), was added. The samples were extracted with dichloromethane (2 ml) under alkaline conditions ($K_2CO_3$, 3 ml) for 30 min. The organic layer was separated and the solvent was completely evaporated. The residue was further characterized by adding MSTFA (N-methyl-N-trimethylsilyl trifluoroamide, 20 μl) and heating the reaction mixture at 70°C for 15 min. The silylethers obtained were quantified by high resolution gas chromatography as described above. The limit of determination was 100 ng per ml of urine, with the precision of 9% at the level of 5000 ng/ml.

*Trans-3′-hydroxycotinine glucuronide* - The total concentration of *trans*-3′-hydroxycotinine was determined after enzymatic hydrolysis of urine samples. To 0.5 ml of urine, which was diluted with an equal amount of sodium acetate buffer (0.05 M, pH 4.7), was added β-glucuronidase from *Helix pomatia* (6000 U) dissolved in sodium acetate buffer (0.2 ml). After incubation at 37°C for 24 h, the reaction mixture was analyzed for the total concentration of *trans*-3′-hydroxycotinine and the concentration of glucuronic acid conjugate was calculated from these data (20).

*Nicotine-N′-oxide and cotinine-N-oxide* - To samples of urine (0.5 ml), the reduction reagent titanium chloride (200 mg/ml of conc. HCl, 0.2 ml) was added. After reaction at room temperature for 2 h, the mixture was rendered alkaline with sodium hydroxide (5M, 2 ml) and then analyzed using the procedure described for nicotine and cotinine.

## Results

### Subjects

Age distribution and consumption data for users of *snus* and chewing tobacco are illustrated in Table 1. The average age was almost the same for loose (38.8 yr) and portion-bag users (40.8 yr), while on average the users of chewing tobacco were older (50.4 yr).

The amount of tobacco product consumed daily differed among the three groups of smokeless tobacco users. The average consumption was higher for users of loose (20.8 g/day) than for users of portion-bag *snus* (14.4 g/day), but only the difference in consumption between users of chewing tobacco (7.2 g/day) and *snus* users was found to be statistically significant. The tobacco was kept in the mouth for about the same number of hours a day within all three groups of smokeless tobacco users, the average values ranging within 12.3 to 13.1 h.

The number of years with regular habit was significantly higher for the users of loose *snus* (14.5 yr) than for the users of portion-bag *snus* (7.4 yr), while the users of chewing tobacco (9.5 yr) did not differ significantly from the *snus* users.

### Analysis of chemical constituents in Swedish smokeless tobacco products

Both portion-bag *snus* and loose *snus* are manufactured from ground tobacco using a heat-treatment process. During this process most volatile nitrosamines are distilled off, which is reflected by low concentrations in Swedish products (21). In contrast to the fermentation process commonly used in the USA, no tobacco-specific nitrosamines (TSNA) are formed during processing.

The levels of TSNA and nicotine were about the same in portion-bag and loose *snus*, while the chewing tobacco product contained about twice as much nicotine per g product and considerably smaller amounts of nitrosamines than the *snus* products. The pH of the *snus* products was alkaline (7.9–8.6) and about 0.5 units higher in loose *snus* than in portion-bag *snus*. The pH of the chewing tobacco product was similar to that of pure tobacco (i.e. slightly acidic) as no changes of pH occurred during processing (Table 2).

### Clinical examination

The most common way to use *snus* in Sweden is to deposit 1–2 g of loose *snus*

Table 1. Age distribution and consumption data for *snus* users and chewers (average ± s.d. and range)

| Users of smokeless tobacco | Mean age (years) | Amount/day (grams) | Time/day (hours) | Duration (years) |
|---|---|---|---|---|
| Portion-bag moist *snus* | 40.8 ± 8.7 | 14.4 ± 7.1 | 13.1 ± 3.1 | 7.4 ± 6.6 |
| ($n$ = 23) | (21–57) | (5.8–32.8) | (8.0–20.0) | (3.5–35.0) |
| Loose moist *snus* | 38.8 ± 13.8 | 20.8 ± 15.5 | 12.3 ± 3.6 | 14.5 ± 6.3 |
| ($n$ = 22) | (22–75) | (6.7–82.4) | (6.0–16.0) | (5.0–29.0) |
| Chewing tobacco | 50.4 ± 9.6 | 7.2 ± 4.0 | 13.0 ± 4.0 | 9.5 ± 6.7 |
| ($n$ = 9) | (38–68) | (1.9–12.7) | (7.5–17.0) | (3.0–24.0) |


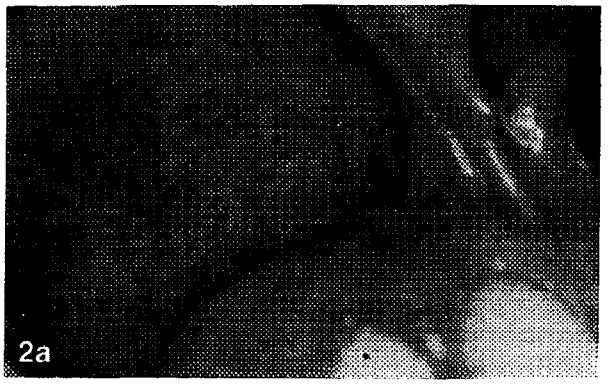



*Fig. 1.* Man, 39 yr, showing a clinical Degree 3 lesion. Tobacco exposure data: loose *snus* of Brand A used daily for 3 yr, 16 g daily over 10 h. Placing of the quid: 13–11 region.

*Fig. 2a, b.* Same patient as in Fig. 1. Follow-up after 3 yrs. Healthy mucosa is seen at the site where the *snus* was previously placed (2a), and leukoedema is present in the buccal mucosa (2b). Tobacco exposure data: portion-bag *snus* of the Brand A used daily for 3 yr, 16 g daily over 10 h. Placing of the quid: at different sites in the vestibular area.

or a pouch of portion-bag *snus* (1 g) in the vestibular area inside the upper lip. Among the 22 users of loose *snus* were recorded 1 (4%) Degree 1, 5 (23%) Degree 2 and 16 (73%) Degree 3 lesions in the mucosa at the site where the *snus* was regularly placed. The corresponding data for the 23 portion-bag users were 2 (9%) subjects with Degree 1, 11 (48%) with Degree 2, 9 (39%) with Degree 3 lesions and 1 (4%) subject with only leukoedema. The portion-bag user whose buccal mucosa showed just slight leukoedema had, at a previous examination 3 years earlier, been using loose *snus* and had done so for 3 years with a daily consumption of 16 g/day during 10 h/day. At that time, a clinical Degree 3 lesion was recorded in the vestibular area (between the canines), where the *snus* was placed (Fig. 1). After the first clinical examination, he changed from loose to portion-bag *snus* of the same brand. He maintained the same daily consumption, but now he placed the pouch of *snus* at many different sites. At the present examination, the mucosa where a Degree 3 lesion was previously recorded had healed completely (Fig. 2a) and no specific lesions other than leukoedema in the buccal mucosa were recorded (Fig. 2b).

Table 2. Analytical data on Swedish smokeless tobacco products

| Product | Nicotine (mg/g) | TSNA (ug/g) | pH |
|---|---|---|---|
| Portion-bag *snus* | | | |
| Brand A | 9.0 | 5.7 | 8.2 |
| Brand B | 10.0 | 6.0 | 7.9 |
| Brand C | 9.2 | 5.5 | 8.1 |
| Brand D | 10.3 | 3.7 | 8.0 |
| Loose *snus* | | | |
| Brand $A_1$ | 9.0 | 7.7 | 8.5 |
| Brand $B_2$ | 9.1 | 6.1 | 8.6 |
| Brand $C_1$ | 8.6 | 7.0 | 8.5 |
| Chewing tobacco | | | |
| Brand E | 21.2 | 1.8 | 4.9 |

Based on close to equal daily consumption (hr/day, g/day) and type of *snus* brand, it was possible to identify 9 matched pairs of loose and portion-bag users (Table 3). Two pairs (Nos 1, 2) showed clinical Degree 2 lesions and two pairs (Nos 8, 9) showed clinical Degree 3 lesions. In the other five pairs, the loose *snus* users had a Degree 3 lesion and the portion-bag users a Degree 2 lesion. Within each pair (except Nos 7, 8), loose and portion-bag users had similar doses of nicotine. However, no correlation could be found between the total dose of nicotine and any of the consumption factors (h/day and g/day).

Chewing tobacco was in many cases used the same way as moist snus (i.e. positioned in the upper buccal cavity). Seven of the 9 users of chewing tobacco showed leukoedema in the buccal mucosa. In addition to this finding, six subjects who placed the tobacco on a permanent site, either without or after chewing it, had clinical lesions at the site where the tobacco was placed. Four of them had a Degree 2 and two a Degree 1 lesion.

#### Exposure and uptake data

The degree of nicotine extraction as well as the amount of nicotine extracted during 24 h were significantly higher among the tobacco users of chewing tobacco and loose *snus* than among the portion-bag *snus* users (Table 4). The high amount of extracted nicotine in the group of loose *snus* users was due to the high degree of extraction as well as to

Table 3. Nine matched pairs of users of loose (L) and portion-bag (P) *snus* according to daily consumption

| Pair No. | Subject | Age (year) | Type of snus | Hours/day | G/day | Clinical degree | Systemic dose (mg/24 h) |
|---|---|---|---|---|---|---|---|
| 1 | 274 | 36 | L | 8.5 | 9.1 | 2 | 20.7 |
| | 022 | 38 | P | 10.0 | 10.3 | 2 | 23.2 |
| 2 | 309 | 33 | L | 14.0 | 7.8 | 2 | 38.7 |
| | 048 | 52 | P | 14.5 | 7.2 | 2 | 32.4 |
| 3 | 267 | 24 | L | 12.0 | 25.1 | 3 | 18.1 |
| | 054 | 28 | P | 10.5 | 26.2 | 2 | 20.0 |
| 4 | 272 | 44 | L | 13.0 | 6.8 | 3 | 39.0 |
| | 070 | 42 | P | 16.0 | 7.5 | 2 | 35.4 |
| 5 | 334 | 43 | L | 14.5 | 13.4 | 3 | 17.2 |
| | 398 | 32 | P | 13.0 | 14.4 | 2 | 13.4 |
| 6 | 234 | 51 | L | 15.0 | 21.3 | 3 | 54.5 |
| | 015 | 41 | P | 15.0 | 21.2 | 2 | 46.1 |
| 7 | 318 | 25 | L | 16.0 | 24.4 | 3 | 24.4 |
| | 023 | 47 | P | 14.0 | 24.0 | 2 | 66.7 |
| 8 | 236 | 44 | L | 15.0 | 16.0 | 3 | 58.7 |
| | 061 | 37 | P | 14.0 | 13.7 | 3 | 24.8 |
| 9 | 277 | 29 | L | 13.0 | 17.2 | 3 | 34.7 |
| | 041 | 45 | P | 14.0 | 17.4 | 3 | 27.8 |

the high daily consumption. The amount of extracted TSNA differed significantly between the groups. The degree of TSNA-extraction from *snus* was somewhat higher than the extraction of nicotine. The users of loose *snus* extracted on average about three times as much TSNA from the *snus* than the users of portion-bag *snus*. Only small amounts of TSNA were extracted from the chewing tobacco as this product contains low levels of nitrosamines.

At steady-state, the saliva cotinine concentration was strongly correlated to the intake of nicotine (22, 23). As is shown in Table 4, there were no differences in saliva cotinine concentrations between the three groups of smokeless tobacco consumers. The highest individual values were found in the users of chewing tobacco.

The systemic dose (i.e. the total amount of nicotine and metabolites excreted in the urine during 24 h) was significantly higher for the users of chewing tobacco than for the users of *snus*. No difference in systemic dose was found between users of loose and portion-bag *snus*. There was a fairly good correlation between the saliva cotinine concentrations and the systemic dose in the total population studied ($r = 0.6496$, $p < 0.001$), as well as in each group of smokeless tobacco users.

#### Analysis of nicotine and metabolites

In order to compare the relative distribution of metabolites among smokeless tobacco users, the amount of each metabolite was converted to nicotine equivalents. The distribution of urinary nicotine and metabolites as a percentage of the total amount of metabolites excreted during 24 h is illustrated in Table 5. The excretion profiles did not differ between the three groups of smokeless tobacco users. For all three groups the total amount of nicotine (i.e. free and glucuronic acid conjugated nicotine) accounted for approximately 11%, the total amount of cotinine for about 17%, and the total amount of *trans*-3′-hydroxycotinine for about 60%. The nicotine-N′-oxides and cotinine-N-oxide corresponded to 9% and 3% of the total amount of nicotine equivalents excreted in the urine. In the total group of smokeless tobacco users, about 60% of the ingested nicotine was metabolized via phase 1 metabolic pathways, while the resulting 35% underwent glucuronic acid conjugation (phase 2 metabolism). The ratio of N-oxidized over C-oxidized metabolites was about 0.1.

## Discussion

The subjects who participated in this study were selected from a previous investigation (24), aiming at recruiting loose and portion-bag *snus* users with equal daily consumption (day and h/day). Three years had passed since the original study was performed, yielding mean age and average years of duration 3 years higher for the present group of subjects. In the present study, the average daily consumption was 1 to 2 h longer for both group of *snus* users. The loose *snus* users consumed less *snus* per day (20.8 g vs 23.4 g) while the portion-bag users consumed more *snus* per day (14.4 g vs 11.3 g) than in the previous investigation. Both groups of *snus* users consumed about 5 g more than the average consumption registered for users of Swedish loose and portion-bag *snus*, which is 15.7 and 9.3 g respectivey (1).

This study confirmed the previously reported less pronounced clinical changes among portion-bag users as compared to those of loose *snus* (24), despite the fact that in this study the portion-bag users had higher, and loose *snus* users lower, daily consumption than those taking part in the previous

Table 4. Amount of nicotine and TSNA extracted during 24 h (average ± s.d. and range)

| Category of smokeless tobacco product | Extracted nicotine (mg/24 hours) | Degree of nicotine extraction (%) | Extracted TSNA (μg/24 hours) | Degree of TSNA extraction (%) | Saliva cotinine (ng/ml) | Systemic dose (mg/24 hours) |
|---|---|---|---|---|---|---|
| portion-bag *snus* ($n = 23$) | 47.6 ± 31.4 (12.3–164.4) | 37.4 ± 17.6 10.3–74.7 | 44.5 ± 25.7 (10.7–120.3) | 55.7 ± 20.5 (23.7–84.2) | 342.9 ± 180.8 (113.4–612.2) | 34.5 ± 23.1 (12.3–87.6) |
| Loose *snus* ($n = 22$) | 94.7 ± 67.9 (18.5–274.3) | 49.1 ± 17.2 (12.7–81.7) | 125.3 ± 115.5 (19.6–403.8) | 64.1 ± 16.4 (35.2–90.5) | 326.6 ± 135.6 (116.2–589.1) | 35.6 ± 18.6 (9.3–74.3) |
| Chewing tobacco ($n = 9$) | 76.4 ± 45.2 (19.5–153.3) | 54.3 ± 23.9 (16.2–95.5) | 3.3 ± 2.4 (1.1–7.1) | 29.7 ± 11.4 (20.0–50.0) | 470.8 ± 271.1 (58.5–1001.2) | 54.1 ± 32.6 (12.9–124.1) |

Table 5. Distribution of urinary nicotine metabolites in percentage of the total amount of metabolites excreted during 24 h (average ± s.d.)

| Nicotine metabolites | Users of portion-bag *snus* ($n=23$) | Users of loose *snus* ($n=22$) | Users of chewing tobacco ($n=9$) | All smokeless tobacco users ($n=54$) |
|---|---|---|---|---|
| Nicotine | 8.8±5.1 | 9.1±6.2 | 5.1±5.3 | 8.3±5.7 |
| Nicotine-GlcA | 2.2±1.4 | 3.3±2.1 | 2.8±1.9 | 3.0±1.8 |
| Cotinine | 7.6±2.7 | 8.0±2.6 | 8.4±3.2 | 7.9±2.7 |
| Cotinine-GlcA | 8.3±3.7 | 9.3±5.6 | 9.6±4.6 | 8.9±4.6 |
| 3′-Hydroxycotinine | 42.0±9.7 | 39.7±11.7 | 45.2±10.2 | 41.6±10.6 |
| 3′-Hydroxycotinine-GlcA | 20.1±9.6 | 17.6±12.9 | 21.9±9.3 | 19.4±11.0 |
| Nicotine-N′-oxide | 7.9±6.5 | 10.7±7.7 | 5.1±3.7 | 8.6±6.9 |
| Cotinine-N-oxide | 2.7±2.5 | 2.3±2.4 | 2.4±1.6 | 2.5±2.3 |

investigation. The portion-bag users showed predominantly Degree 1 and 2 lesions, while loose *snus* users had more Degree 3 lesions. This is further illustrated in the 9 pairs of loose and portion-bag *snus* users matched according to daily consumption (Table 3). Two clinical Degree 2 lesions and 7 Degree 3 lesions were recorded in the group of loose *snus* users and a reversed clinical pattern was recorded for the group of portion-bag users. The present case report clearly shows that the clinical *snus*-related mucosal change that develops after usage of loose *snus* is reversible and that a change to portion-bag *snus* results in less pronounced clinical changes. Only a small number of users of chewing tobacco participated in the study and their daily exposure to tobacco was comparatively high (7.2 g vs 4.4 g) as compared to those participating in the previous study (16). The clinical changes, leukoedema and clinical Degree 1 and 2 lesions, recorded in the oral mucosa of users of chewing tobacco were in accordance with previous findings.

In epidemiological and clinical studies on the effect of tobacco smoke, environmental tobacco smoke and smokeless tobacco, it is of great importance to quantify the degree of exposure. Attempts have been made to correlate the exposure to tobacco smoke with various biological markers. The concentrations of nicotine and its main metabolite, cotinine, in different body fluids have been considered to be the best measure of tobacco smoke exposure. The best estimate of the nicotine dose is obtained by measuring the concentrations of nicotine and all of its metabolites excreted in urine.

Preliminary results from a mass balance study of nicotine after intravenous injection of 0.4 μg/min/kg body weight of nicotine over 8 h to abstinent tobacco users have shown that nicotine and its seven main metabolites account for about 95% of the administered dose of nicotine. As can be seen in Table 5, there was no difference in metabolic pattern between the three groups of smokeless tobacco users. The average excretion profile for the 54 smokeless tobacco users shows that nicotine and its glucuronide account for 8% and 3%, cotinine and its glucuronide for 8% and 9%, and 3′-hydroxycotinine and its glucuronide for 42% and 19%, respectively. Nicotine-N′-oxides and cotinine-N-oxide account for 9% and 3%, respectively, of the nicotine dose. This profile is in good agreement with that obtained after intravenous injection of nicotine as well as that obtained from smokers (25). These results indicate that the metabolism of nicotine is independent of dose and mode of administration.

Thorough clinical investigations have been performed on soft tissue changes of smokeless tobacco users in Sweden, but they all lack data on exposure to and uptake of tobacco constituents. At steady-state, the rate of excretion of metabolites reflects the generation rate. The daily intake of nicotine by tobacco users could thus be estimated by measuring nicotine and its metabolites in 24-h urine samples. The average intake of nicotine measured as the systemic dose of nicotine was 35 mg per 24 h for users of *snus*, while it was 50% higher for users of chewing tobacco. These values are somewhat higher than those obtained in a study on the uptake of nicotine by cigarette smokers (25).

At steady-state, the concentration of cotinine in plasma is linearly and directly related to the intake of nicotine at levels usually found in smokers (23), as well as to levels found in subjects exposed to environmental tobacco smoke (22). Saliva cotinine concentrations therefore reflect the nicotine intake during habitual usage of smokeless tobacco. The average values for users of loose and portion-bag *snus* were 343 and 327 ng/ml and were found to be similar to those of smokers (26), while the average value for users of chewing tobacco was 50% higher. As this study has shown that there is a good correlation between salivary cotinine concentrations and the systemic dose of nicotine, salivary steady-state concentrations of cotinine could be used to reflect the total intake of nicotine with habitual usage.

Twice as much nicotine was extracted from loose than from portion-bag *snus*. Still, there was no difference in saliva cotinine concentrations and systemic dose of nicotine between the two groups of *snus* users, and accordingly no correlation was found between the systemic dose or the saliva cotinine concentrations and the amount of nicotine extracted from the *snus*. This discrepancy between the amount extracted and the actual uptake of nicotine may be due to the fact that users of loose *snus* have a higher salivary secretion rate and therefore spit or swallow much more saliva than users of portion-bag *snus*.

Nearly three times as much TSNA was extracted from loose as from portion-bag *snus*. As the amount of TSNA and nicotine extracted from the *snus* is well correlated, it may be assumed that the amount of TSNA expectorated was proportional to that of nicotine. This indicates that there might be a higher uptake of TSNA from loose *snus* than from portion-bag *snus*.

The clinical severity of buccal mucosal changes did not correlate with any of the biological markers reflecting the uptake of tobacco constituents. The difference in tissue response between portion-bag users and loose *snus* users found both here and in the previous study was probably due to the pH differences of the two types of products (i.e. the higher the pH of the *snus*, the more severe changes in the mucosa). This is further supported by the fact that users of chewing tobacco, which has considerably lower pH, exhibit only slight changes in the buccal mucosa.

In conclusion this study shows that among this group of smokeless tobacco users:

- The average 24 h intake of nicotine, estimated as nicotine equivalents excreted during 24 h, was 35 mg for *snus* users and 50% higher for users of chewing tobacco (54 mg).
- The average steady-state saliva cotinine concentration was about 300 ng ml for *snus* users, which is similar to

that found in smokers, and was about 50% higher for users of chewing tobacco.

- Users of oral moist snuff (*snus*) portion-bag users exhibit less pronounced clinical changes of the oral mucosa compared to users of loose *snus*. The portion-bag users showed predominantly Degree 1 and 2 lesions, while users of loose *snus* had more Degree 3 lesions. The clinical findings recorded in the oral mucosa of users of chewing tobacco were leukoedema and Degree 1 and 2 lesions.
- The clinical severity of buccal mucosal changes correlated neither with the markers for exposure (i.e. nicotine and TSNA extracted from the smokeless tobacco product), nor with biological markers for uptake of tobacco constituents (i.e. nicotine equivalents excreted during 24 h and saliva cotinine concentrations).
- The average excretion profile of nicotine is:
  Nicotine 8%, nicotine-GlcA 3%, cotinine 8%, cotinine-GlcA 9%, 3′-hydroxycotinine 42%, 3′-hydroxycotinine-GlcA 19%, nicotine-N′-oxide 9% and cotinine-N-oxide 3%.

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