In-vivo extraction of lead, cadmium and Tobacco Specific Nitrosamines from four brands of Swedish ‘snus’ in regular snus users.
**Principal Investigator**

Erik Lunell, M.D., Ph.D.

CROel AB
Slottsvägen 21
SE-252 84 Helsingborg
Sweden

croelab@telia.com
Telephone: +46 46 12 04 60
Fax No: +46 42 913 92

---

**Sponsor**

Margareta Curvall Ph. D.

Swedish Match North Europe
Maria Skolgata 83
SE-118 85 Stockholm
Sweden

margareta.curvall@swedishmatch.se
Telehone: +46 8 658 04 44
Fax No: +46 8 668 97 77

---

Signature                        Date                        Signature                        Date
**Study Director**
Marianne Lunell, Reg. Pharmacist
CROel AB
Slottsvägen 21
SE-252 84 Helsingborg
Sweden

croelab@telia.com
Telephone: +46 46 12 04 60
Fax No: +46 42 913 92

**Statistician**
Fredrik Hansson M. Sc.
HH-Statistik
Planteringsv. 11
SE-244 65 Furulund
Sweden

fredrik.hansson@hh-statistik.m.se
Telephone: +46 46 73 82 87
Telefax: +46 46 73 82 73

**Analyst**
Ingrid Forsblom
Swedish Match North Europe
Maria Skolgata 83
SE-118 85 Stockholm
Sweden

ingrid.forsblom@swedishmatch.se
Telephone: +46 8 6580 406
Fax No: +46 8 6689 777

**Bioanalyst**
Dr Colin Feyerabend
ABS Laboratories, Medical Toxicology Unit
Wardalls Groove, Avonlay Road
London SE14 5ER, United Kingdom

abs.labs@dial.pipex.com
Telephone: +44 171 277 55 72
Telefax: +44 171 277 55 72
TABLE OF CONTENTS
SIGNATURE PAGE ........................................................................................................2

STUDY SYNOPSIS ........................................................................................................5

1. PROTOCOL SUMMARY .........................................................................................7

2. INTRODUCTION .....................................................................................................7

3. STUDY OBJECTIVES ............................................................................................9

4. STUDY DESIGN .....................................................................................................9

5. STUDY SITE AND TIMETABLE ............................................................................9

6. MATERIAL AND METHODS ...............................................................................9

7. ASSESSMENT OF SAFETY ................................................................................13

8. STATISTICS .........................................................................................................13

9. QUALITY CONTROL (QC) AND QUALITY ASSURANCE (QA) .....................14

10. ETHICS ................................................................................................................14

11. DATA HANDLING AND RECORD KEEPING ................................................15

REFERENCES ..........................................................................................................16

Appendices

Appendix 1. DECLARATION OF HELSINKI

Appendix 2. PLASMA SPECIMEN COLLECTION / HANDLING

Appendix 3. PRODUCT ACCOUNTABILITY

Appendix 4. PRODUCT COMPLAINTS

Appendix 5. SHEDULE OF ACTIVITIES

Appendix 6. INFORMED CONSENT
STUDY SYNOPSIS

Study code: SM WS03

Title: Study of the *in-vivo* extraction of lead, cadmium and nitrosamines from four brands of Swedish ‘snus’ in regular snus users.

Objectives: To estimate the *in-vivo* extraction of lead, cadmium and tobacco specific nitrosamines (TSNAs) from Swedish snus.

Total sample size: 32 male healthy regular snus users.

Study design: Open label, randomised, four-way single dose study.

Subject population criteria: 18-50 years old, male non-smoking subjects, regularly using ≥ 7 portions of snus daily since minimum 1 year.

Test articles: A= “General Portion” 1 g portion snus containing approximately 8 mg nicotine per portion. Batch No.: B= “Catch Licorice Portion” 1 g portion snus containing approximately 8 mg nicotine per portion. Batch No.: C= “Catch Licorice Portion Mini” 0.5 g portion snus containing approximately 4 mg nicotine per portion. Batch No.: D= “Catch Dry Licorice Portion Mini” 0.3 g portion snus containing approximately 4 mg nicotine per portion. Batch No.: 

Procedure: The “General”, “Catch”, “Catch Mini” and “Catch Dry Mini” snus portions will be administered once every hour (4 administrations/brand) and will be kept between the upper lip and the gum for 30 minutes. Preload of own brand each morning.

Snus sampling: Each portion of used snus will be collected and frozen (-20 °C) pending analysis of lead (Pb), cadmium (Cd) and tobacco specific nitrosamines (TSNAs). Unused snus collected and deep frozen for analysis and calculation of extracted dose.

Study parameters: Extracted amount of lead, cadmium and TSNAs, respectively, from each portion of snus.

Analysis: One portion is collected for analysis of lead, one for cadmium, one for nicotine and 1 portion for analysis of TSNAs. Frequency distributions of extracted amount of lead, cadmium, nicotine and TSNAs, respectively, will be shown for each type of snus. Catch Mini has displayed the highest extraction of nicotine. Thus extraction will be statistically assessed using Catch Mini as reference.

Statistical considerations: The analyses will be carried out according to a randomised four-way cross-over design, i.e. as differences within in each patient between the four snus preparations. General (A) is the leading brand. Catch Mini (C) has displayed the highest extraction of nicotine. Thus extraction will be statistically assessed using Catch Mini as reference. There will be analyses performed in order to ensure that no carry-over effect exists (analysis of variance).
Sample size calculation: The primary endpoint is to assess if A and C are equivalent regarding the Cadmium extraction. Former studies have indicated that both A and C would have a 30% extraction of the baseline Cadmium level. In order to declare A and C as equivalent the extraction should not differ by more than 30% with a 90% confidence interval and a power of 80%. Using a formula from Machin et al. the number of patients needed to show this equivalence be 29. Thirty two subjects will be included to compensate for possible withdrawals.

Study site: CROel AB, Helsingborg, SWEDEN
1. PROTOCOL SUMMARY

In an open label, randomized, two-way cross-over study, 32 male healthy regular snus users will be given repeated doses of four different types of portion snus: “General”, “Catch”, “Catch Mini” and “Catch Dry Mini”. Each portion of used snus will be collected and frozen (-20 °C) pending analysis of lead (Pb), cadmium (Cd), nicotine and tobacco specific nitrosamines (TSNAs). Unused snus is collected and deep frozen for analysis and calculation of extracted dose. Calculations of extracted amount of lead, cadmium, nicotine and tobacco specific nitrosamines (TSNAs) respectively, will be done for each type of snus. Catch Mini has displayed the highest extraction of nicotine. Thus extraction will be statistically assessed using Catch Mini as reference.

2. INTRODUCTION

2.1 Background

Tobacco contains nitrate that is microbially activated to nitrite, which may react with alkaloids to cancer causing tobacco specific nitrosamines (TSNAs) during curing and storage of the tobacco. Different selection and curing methods can affect the levels of nitrites and hence TSNAs in the raw tobacco before processing (1). Swedish snus manufacturers have selected tobacco blends that have been air and sun cured (dried), while US moist snuff products include blends containing fire cured tobacco. After curing, raw cured tobacco is grinded, and sifted before processing. Ingredients added to Swedish snus are: 20-60% water, 1.5-3.5% sodium chloride, 1.5-3.5% humectants, 1.2-3.5% sodium bicarbonate, and less than 1% flavours. Snus production in Sweden includes a heating process in which the tobacco is treated with steam for 24-36 hours, reaching temperatures of approximately 100°C. The heating process kills bacteria, producing a nearly sterile product. The final product is packaged in cans that are kept in refrigerators during storage, in Sweden also by the retailers. One study examined levels of carcinogenic TSNAs in snus kept at temperatures ranging from -20°C to +23°C over 20 weeks (2). This storage at a variety of temperatures did not produce a significant increase in concentrations of TSNAs, suggesting that the exposure to heat during manufacturing may prevent microbial activation of nitrites to nitrosamines (3).

Because of the differences in manufacturing and storage, Swedish snus has been claimed to contain lower levels of some harmful substances than many of the brands available in North America and notably lower levels than exist in the smokeless tobacco used in the Sudan and India (3). The Swedish manufacturing process contrasts with the one used in the USA, in which the moist snuff products are fermented rather than being subject to high temperatures, allowing a continued formation of TSNAs. In addition, North American smokeless tobacco is usually not stored in refrigerators. One study found that nitrite and TSNA levels increased significantly in US snuff stored at 37°C over four weeks (4).

When studying the content and particularly the in-vivo extraction of harmful substances it is important to make the estimates in relation to the nicotine extracted. Only nicotine in the free-base form is rapidly absorbed through the mucosal membrane, and the proportion of free-base nicotine available from tobacco is determined by the pH level. Although different products
vary in their pH levels, Swedish snus typically has a pH in the range 7.8-8.5 (5,6).
Brunnemann and Hoffmann compared two brands and found only 1% of the nicotine in the
free-base form available for absorption in one brand with a pH of 5.84 while another brand
with a pH of 7.49 had 59% of the nicotine in free-base form (7). Another study found that
a leading Swedish snus brand had a higher pH (and therefore probably more efficient nicotine
delivery) compared with five brands of US smokeless tobacco (8).

Content of harmful substances. A number of studies (6, 8-11) of TSNA content of several
brands marketed in different countries have been published. The total TSNA concentration
varied greatly among the US brands from 4.1 for to 128 (µg/g dry tobacco). There is little
evidence to that TSNA levels in North American snuff have consistently dropped over the
past decade. For example, Copenhagen brand in 1994 had a measured TSNA content of
17.2µg/g and in 2000 it was 41.1µg/g. Snus brands selected in Sweden from 1990, 1991, and
2000 were generally lower and varied from 9.2 to 11.2 µg/g in samples in 1990-91 and 2.8
µg/g in 2000. Brunnemann and Hoffmann (6) examined the effects of storage over six months
at room temperature and found that in the two leading US brands, the TSNA levels increased
by 30-130%, whereas in the Swedish snus brand there was no increase. The manufacturer
of snus has published a voluntary quality standard, the Gothiatek standard," of maximum
permissible limits for "undesirable substances" for its snus products (12). See Table 2.

Table 2. Gothiatek standard. Quality standard for snus products by Swedish Match

| Toxin                              | Limit  
|------------------------------------|--------
| Nitrite                            | 3.5 mg/kg |
| Tobacco specific nitrosamines (TSNA) | 5 mg/kg |
| N-Nitrosodimethylamine (NDMA)      | 5 µg/kg |
| Benz(a)pyrene (BaP)                | 10 µg/kg |
| Cadmium (Cd)                       | 0.5 mg/kg |
| Lead (Pb)                          | 1.0 mg/kg |
| Arsenic (As)                       | 0.25 mg/kg |
| Nickel (Ni)                        | 2.25 mg/kg |
| Chromium (Cr)                      | 1.5 mg/kg |

mg/kg, thousandth gram per kilogram product (based on Snus with 50% water content); µg
/kg, millionth gram per kilogram product (based on Snus with 50% water content); double the
limits for dry weight equivalents.

One method of assessing the potential harmfulness of a tobacco product is to measure the
level of circulating mutagens in body fluid after exposure. Curvall and colleagues (13)
compared the mutagenic activity of urine from snus users, cigarette smokers, and non-tobacco
users. Smokers showed notably increased urinary mutagenic activity, whereas there was no
significant difference between snus users and non-tobacco users.

2.2 Study rationale

The contents of lead (Pb), cadmium (Cd) and tobacco specific nitrosamines (TSNAs) in moist
snuff are well studied (6-11). Their extraction in-vivo, however, is less studied. A
documentation of the extraction of lead (Pb), cadmium (Cd) and tobacco specific nitrosamines
(TSNAs) from various brands of snus therefore appears well motivated.
3. STUDY OBJECTIVES

The objectives of the present study are to estimate the in-vivo extraction of lead, cadmium and tobacco specific nitrosamines (TSNAs) and nicotine from four brands of Swedish snus. The primary objective is to compare the in-vivo extraction of cadmium from General versus Catch Mini snus. Secondary objectives are comparisons of lead and TSNAs.

4. STUDY DESIGN

The study has a randomized, cross-over design, and is an open testing of 4 different types of portion snus. Thus the study comprises a total of four sessions for each subject. Statistic power calculation showed a necessary sample size of 29 subjects. Thirtytwo subjects will participate in the study.

5. STUDY SITE AND TIMETABLE

The study will be performed at CROel AB, Helsingborg, SWEDEN, during 2004. The analysis of cadmium and lead in snus samples before and after usage will be performed by the consultant laboratory AnalyCen AB, Lidköping, Sweden. The analysis of nicotine and tobacco specific nitrosamines will be carried out be the Research Department, Swedish Match North Europe.

6. MATERIAL AND METHODS

6.1. Subjects

Thirtytwo male non-smoking healthy volunteers, regularly using ≥ 7 portions snus daily since minimum 1 year will be selected for participation in the study. They should have no history of cardiac, kidney or hepatic disease, alcohol abuse or drug dependence. A health declaration and interview by the responsible physician shall rule out any disease. The subjects do not have to abstain from nicotine prior to the experimental sessions.

6.1.1. Screening phase/procedures

All subjects will after giving informed consent be interviewed and fill in a health declaration prior to the inclusion to the study. The health declaration will include the following:

- personal data
- previous history of psychiatric, neurological or serious somatic disorders that may interfere with the study
- use of long-term medication
- previous history of allergic reactions
- a self-estimate of the subject’s general physical fitness
6.1.2. Inclusion criteria

1. Male non-smokers, 18 to 50 years of age.
2. Habitual use of ≥ 7 portions snus daily since minimum 1 year.
3. Healthy according to the health declaration and interview.
4. Written informed consent given.

6.1.3 Exclusion criteria

2.2.1.1 Concurrent participation in another clinical trial.
2.2.1.2 History of allergy.

6.1.4 Admission to study

A subject is eligible for admission to study if inclusion criteria are fulfilled and if no exclusion criteria are present as verified by the investigator.

6.1.5 Subject identification

As subjects are included they will get a number between 1 and 32. Each subject included in the study will be uniquely identified by this number and the subject’s initials, which will appear on all study documents.

6.1.6 Subject recruitment

The subjects will mainly be students recruited from the University, Lund, Sweden.

6.2 Study products

6.2.1 Study products

A. “General Portion” 1 g containing approximately 8 mg nicotine per portion. Released nicotine dose approximately 2.4 mg/portion (31%).
Batch No.: 

B. “Catch Licorice Portion” 1 containing approximately 8 mg nicotine per portion.
Released dose approximately 1.8 mg/portion (22%).
Batch No.: 

C. “Catch Licorice Portion Mini” 0.5 g containing approximately 4 mg nicotine per portion.
Released dose approximately 1.8 mg/portion (44%).
Batch No.: 

D. “Catch Dry Licorice Portion Mini” 0.3 g containing approximately 4 mg nicotine per portion. Released dose approximately 0.9 mg/portion (22%).
Batch No.: 
6.2.2 Randomization procedure

The snus treatments will be given according to a computer generated randomization list.

6.2.3 Packaging, labeling and storage

The various types of snus will be delivered in their original packs as delivered from Swedish Match, Stockholm, Sweden. Individual packaging according to a computer generated randomization list will be made. Labeling will be in Swedish. The snus will be stored in a refrigerator (+2 - +8 °C).

Each pack will be labeled (in Swedish):

Snus, containing 8mg, 8 mg, 4 mg and 4 mg nicotine, respectively.

For clinical trial.

CTN: SM WS03
Subject No.: 1 (32)
Treatment.: A (B, C, D)
Batch No.:
Expiry date:
Dosage: according to physician’s instruction
Responsible investigator: Erik Lunell, M.D.
Keep out of reach of children.
Packed on the: Initials:

6.2.4 Product accountability

The test articles will be ordered from CROel HB, Helsingborg, Sweden. After packaging and labeling the articles will be delivered to the Investigator in due time before start of the study. The snus will be delivered in its original container. A ”confirmation of receipt note” will be completed. All unused test articles will be returned at study termination to Swedish Match, Stockholm, Sweden.

6.3 Treatment

The treatments will be given as multiple doses, one sachet administered every hour from 8.00 a.m. to 12.00 (1st and 3rd session) and 14.00 to 18.00 p.m. (2nd and 4th session). A total of four sachets per session will be given. Only non-smoking personnel may perform practical functions in this study. Snus will be used under standardized conditions and executed as follows: One sachet will be placed and kept in the same place between the upper lip and the gum for 30 minutes. PRELOAD???? Nicotine is a potent local vasoconstrictor. The subjects are therefore requested to administer one portion of their regular brand of snuff before leaving home in the morning of each study day, in order to eliminate any discrepancies between the first dose and subsequent doses of snuff.

6.4 Concomitant therapy

There will be no restrictions as to the use of OTC drugs, however the participants are requested to report such use, which will be recorded on the CRF. No other drug under investigation is
allowed concomitantly with the study drug. The subjects are not allowed to participate concurrently in any other study.

6.5. Residual content of Cadmium (Cd) in used snus

Each designated used portion of snus of each preparation will be placed in a sealed container, labelled with a unique number, frozen and stored at -20°C until analysed for Cd content. Ten sachets of unused snus will also be analysed. Mean Cd content of these sachets will be used for the calculation of the extraction of Cd. Analysis of cadmium will be performed under the responsibility of the Research Department, Swedish Match, Stockholm.

6.6. Residual content of lead (Pb) in used snus

Each designated used portion of snus of each preparation will be placed in a sealed container, labelled with a unique number, frozen and stored at -20°C until analysed for Pb content. Ten sachets of unused snus will also be analysed. Mean Pb content of these sachets will be used for the calculation of the extraction of Pb. Analysis of Pb will be performed under the responsibility of the Research Department, Swedish Match, Stockholm.

6.7. Residual content of tobacco specific nitrosamines (TSNAs) in used snus

Each used portion of snus of each preparation will be placed in a sealed container, labelled with a unique number, frozen and stored at -20°C until analysed for TSNAs. Ten sachets of unused snus will also be analysed. Mean content of TSNAs in these sachets will be used for the calculation of the extraction of TSNAs. Analysis of residual TSNAs will be performed at the Research Department, Swedish Match, Stockholm.

6.8. Residual nicotine in used snus

Each used portion of snus of each preparation will be placed in a sealed container, labelled with a unique number, frozen and stored at -20°C until analysed for nicotine content. Ten sachets of unused snus will also be analysed. Mean nicotine content of these sachets will be used for the calculation of extracted dose of nicotine. Analysis of residual nicotine will be performed at the Research Department, Swedish Match, Stockholm.

6.9 Calculation

Extracted amount of the various contents analysed will be calculated for each individual and each analytical result as follows:

\[
\text{Extracted amount} = \text{mean of 10 unused sachets} - \text{residual amount}
\]

Extracted amount of the various contents will be given as absolute amount as well as in relation to the extraction of nicotine for each individual. Nicotine intake (14) as well as resulting plasma levels (15) have been reported.
7. ASSESSMENT OF SAFETY

7.1 Definition

An adverse event (AE) is any untoward medical occurrence in a patient or trial subject administered a drug or biologic (medicinal product) or using a medical device; the event does not necessarily have a causal relationship with that treatment or usage.

7.2 Description of and Recording Instructions for Adverse Events (AE)

Adverse events are to be recorded in the case report forms as specified. If required on the adverse event case report forms, the investigator will use the adjectives MILD, MODERATE, or SEVERE to describe the maximum intensity of the adverse event.

For purposes of consistency, these intensity grades are defined as follows:

- **MILD**
  - Does not interfere with subject's usual function.

- **MODERATE**
  - Interferes to some extent with subject's usual function.

- **SEVERE**
  - Interferes significantly with subject's usual function. Note the distinction between the gravity and the intensity of an adverse event. **Severe** is a measure of intensity; thus, a **severe** reaction is not necessarily a **serious** reaction. For example, a headache may be severe in intensity, but would not be classified as serious unless it met one of the criteria for serious events listed above.

Follow-Up of Adverse Events

All adverse events should be followed until they are resolved or the subject’s participation in the trial ends. Instructions for reporting changes in an ongoing adverse event during a subject's participation in the trial are provided in the instructions that accompany the adverse event case report forms.

In addition, all serious adverse events should continue to be followed even after the subject's participation in the trial is over. Such events should be followed until they resolve or until the investigator assesses them as “chronic” or “stable.” Resolution of such events is to be documented on the appropriate follow-up CRF.

8. STATISTICS

8.1 Randomisation

The snus preparations will be given randomly according to a computer generated list.

8.2 Statistical considerations

The analyses will be carried out according to a randomised four-way cross-over design, i.e. as differences within in each patient between the four snus preparations. General (A) is the leading brand. Catch Mini (C) has displayed the highest extraction of nicotine. Thus extraction will be statistically assessed using Catch Mini as reference.
There will be analyses performed in order to ensure that no carry-over effect exists (analysis of variance). All variables which are continuous will be presented using descriptive statistics such as mean, sd, median, min, max, etc. Variables that are categorical will be presented using frequency tables.

8.3 Sample size calculation

The primary endpoint is to assess if A and C are equivalent regarding the Cadmium extraction. Former studies have indicated that both A and C would have a 30% extraction of the baseline Cadmium level. In order to declare A and C as equivalent the extraction should not differ by more than 30% with a 90% confidence interval and a power of 80%. Using a formula from Machin et al. the number of patients needed to show this equivalence be 29.

\[
N = \frac{2(Z_{\alpha} + Z_{\beta})^2}{\varepsilon^2} \left[ \pi(1-\pi) \right]
\]

Thirty two subjects will be included to compensate for possible withdrawals.

All adverse events will be presented by the different snus flavours and if the amount of adverse events should be high, treatment emergent signs and symptom approach.

9. QUALITY CONTROL (QC) AND QUALITY ASSURANCE (QA)

Monitoring visits to the trial site will be made periodically during the trial, to ensure that all aspects of the protocol are followed. The subject chart and other documents will be reviewed for verification of agreement with data on Case Report Forms. The trial site may also be subject to quality assurance audit by monitor, auditor(s) as well as inspection by appropriate regulatory agencies. The investigator guarantees access to CRFs, subject charts and all relevant documents by monitor, auditor and appropriate regulatory agencies. It is important that the investigator and relevant personnel are available during the monitoring visits and audits and that sufficient time is devoted to the process.

10. ETHICS

10.1 Ethical Conduct of the Trial

The trial will be performed in accordance with the recommendations guiding physicians in biomedical research involving human subjects adopted by the 18th World Medical Association Assembly, Helsinki, Finland, 1964 (Declaration of Helsinki) and later revisions (See Appendix 1). The trial will be consistent with Good Clinical Practice (GCP) and applicable regulatory requirements.

10.2 Ethics Committee (EC)

It is the responsibility of the investigator to obtain approval of the trial protocol/amendments from EC. The investigator should file all correspondence with the EC. Copies of EC approvals should be forwarded to Swedish Match. Since the study products fall under the Swedish food legislation and no medicinal drug is under investigation in the present study no application or notification of the Swedish Medical Agency (MPA) will be done.
10.3 Subject Information and Consent

It is the responsibility of the investigator to give each subject prior to inclusion in the trial, full and adequate verbal and written information regarding the objective and procedures of the trial and the possible risks involved. The subjects must be informed about their right to withdraw from the trial at any time. Written subject information (a proposed informed consent is attached as appendix 6) must be given to each subject before enrolment. The written subject information must not be changed without prior discussion with Swedish Match and approval by the EC. Furthermore, it is the responsibility of the investigator to obtain signed informed consent from all subjects prior to inclusion in the trial.

10.4 Stopping rules/Discontinuation Criteria

Swedish Match reserves the right to discontinue the trial prior to inclusion of the intended number of subjects, but intends only to exercise this right for valid scientific or administrative reasons. After such a decision, the investigator must contact all participating subjects within one week.

11. DATA HANDLING AND RECORD KEEPING

11.1 Data Management

A Case Report Form (CRF) should be completed for each included subject. Before data entry, the CRF’s will be checked for completeness and accuracy. If data are missing the reason must be given in the CRF. The data will be compiled into an analytical report and a computer file by the responsible analyst. The responsible statistician will transfer data from the computer files for a statistical analysis and will compile the result into a statistical report. These data will be used by the Investigator who writes the final report.

11.2 Case Report Forms

The completed original CRFs are the sole property of Swedish Match and should not be made available in any form to third parties, except for authorized representatives of appropriate Health/Regulatory Authorities, without written permission from Swedish Match.
REFERENCES


