

# Quantitation of Metabolites of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone after Cessation of Smokeless Tobacco Use<sup>1</sup>

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## ABSTRACT

Two major metabolites of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were previously shown to be highly persistent in human urine after cessation of cigarette smoking. We hypothesized that NNK or its metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), was sequestered in the lung. In this study, we further evaluated this hypothesis by quantifying the NNK metabolites, NNAL and its glucuronides (NNAL-Gluc), in urine and plasma after cessation of smokeless tobacco use, in which NNK is administered p.o. rather than by inhalation. Thirteen male nonsmokers, 11 snuff dippers and 2 tobacco chewers, participated in the study. Urine and plasma were obtained at baseline and at intervals 2–126 days after cessation of smokeless tobacco use. The distribution half-lives  $t_{1/2\alpha}$  (days) of NNAL ( $1.32 \pm 0.85$  versus  $3.35 \pm 1.86$ ) and NNAL-Gluc ( $1.53 \pm 1.22$  versus  $3.89 \pm 2.43$ ) were significantly shorter in smokeless tobacco users than in smokers. There were no significant differences in the terminal half-lives  $t_{1/2\beta}$  (days) of NNAL ( $26.3 \pm 16.7$  versus  $45.2 \pm 26.9$ ) and NNAL-Gluc ( $26.1 \pm 15.1$  versus  $39.6 \pm 26.0$ ) in smokeless tobacco users and smokers. Baseline levels as well as renal clearance of the NNK metabolites correlated with number of tins or pouches of smokeless tobacco consumed. Ratios of (S)-NNAL:(R)-NNAL and (S)-NNAL-Gluc:(R)-NNAL-Gluc in urine were significantly (3.1–5.7 times) higher 7 days after cessation than at baseline in both smokeless tobacco users and smokers, indicating stereoselective retention of (S)-NNAL. Collectively, the results of this study suggest that there is a receptor in the human body, possibly in the lung, for (S)-NNAL, the more carcinogenic NNAL enantiomer. These data may have considerable implications for understanding mechanisms of tumor induction by NNK.

## INTRODUCTION

The tobacco-specific NNK<sup>3</sup> (Fig. 1) is a potent pulmonary carcinogen in rodents, inducing lung tumors in rats, mice, and hamsters (1). In rats in particular, low doses of NNK readily produce tumors (2, 3). Voluminous data have conclusively established the presence of NNK in both tobacco and tobacco smoke (4–7). It is routinely detected in quantities of 1–2  $\mu\text{g/g}$  of tobacco in products such as moist snuff, whereas the amounts in cigarette smoke are 100–200 ng/cigarette. Collectively, these data support the proposal that NNK plays a significant role as a cause of lung cancer in people exposed to tobacco products (8).

One of the major pathways of NNK metabolism is conversion to its carbonyl reduction product NNAL (Fig. 1; Ref. 1). NNAL is also a potent pulmonary carcinogen in rats and mice (1). NNAL is detoxified

by glucuronidation, and NNAL-Gluc (Fig. 1) is excreted in urine. NNAL and NNAL-Gluc are detected in the urine of smokers, smokeless tobacco users, and nonsmokers exposed to environmental tobacco smoke (1). NNAL and NNAL-Gluc are reliable biomarkers of NNK uptake in humans (1, 9). The metabolic activation of NNK proceeds by hydroxylation of the carbons adjacent to the *N*-nitroso group ( $\alpha$ -hydroxylation), producing DNA adducts that are involved in cancer induction by NNK and NNAL (1).

In an earlier study, we quantified urinary NNAL and NNAL-Gluc in people who had stopped smoking (10). The results demonstrated that NNAL and NNAL-Gluc were remarkably persistent in the body. One week after smoking cessation, 34.5% of the baseline NNAL plus NNAL-Gluc was detected in urine, whereas the corresponding value for cotinine, the major metabolite of nicotine, was 1.1%. Even 6 weeks after smoking cessation, 7.6% of the baseline NNAL plus NNAL-Gluc levels were still detected in urine. These results suggested the presence of a high-affinity compartment where NNK or NNAL was sequestered and slowly released. We hypothesized that this compartment may be in the lung, based on a recent study in rats indicating that lung tissue specifically sequestered NNAL at prolonged times after NNK dosing.<sup>4</sup>

The presence of NNK in both unburned tobacco and tobacco smoke allowed us to design a study to further investigate our hypothesis. In smokers, NNK is administered mainly by inhalation. The “first-pass” exposure of the lung to NNK or NNAL would be much greater compared with that in smokeless tobacco users, to whom NNK is administered p.o. Thus smokers would receive a much higher initial pulmonary dose of NNK or NNAL. If the sequestering compartment were in the lung, we might expect greater retention of NNK or NNAL in smokers than in smokeless tobacco users. Therefore, in this study, we quantified urinary NNAL and NNAL-Gluc after cessation of smokeless tobacco use. Types of oral smokeless tobacco commonly used in the United States include chewing tobacco and moist snuff (11, 12). Chewers extract tobacco juice from a plug, twist, or loose-leaf product, whereas snuff dippers place a pinch of moist tobacco product between their cheek and gum. Snuff dipping is a known cause of oral cavity cancer (11). Unfortunately, there has been a considerable resurgence in the popularity of snuff dipping in the United States, where it is commonly practiced by young males (12).

## MATERIALS AND METHODS

**Study Design.** The study was approved by the University of Minnesota Research Subjects' Protection Programs Institutional Review Board Human Subjects Committee. Smokeless tobacco users were recruited through metropolitan newspaper advertisements. Subjects were initially screened by telephone. They must have been users of smokeless tobacco for at least 3 years. The subjects used smokeless tobacco at their normal rate for 7 days prior to their quit date. During this period, two baseline 24-h urine collections and one blood sample were taken. Subjects reported to the University of Minnesota Tobacco Research Programs clinic on the evening before their quit date. Data

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<sup>3</sup> The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNAL-Gluc, a mixture of 4-(methylnitrosamino)-1-(3-pyridyl)-1-(*O*- $\beta$ -D-glucopyranuronosyl)butane (NNAL-*O*-Gluc) and 4-(methylnitrosamino)-1-(3-pyridyl)-*N*- $\beta$ -D-glucopyranuronosyl)-1-butanololium inner salt (NNAL-*N*-Gluc); CSP-GC-TEA, chiral stationary-phase gas chromatography with nitrosamine-selective detection; HPLC, high-pressure liquid chromatography.

<sup>4</sup> Z. Wu, P. Upadhyaya, S. G. Carmella, S. S. Hecht, and C. L. Zimmerman, submitted for publication.

were collected on vital signs, concomitant medications, and self-reported tobacco use. Subsequently, the subjects provided 24-h urine collections and blood samples 2, 7, 21, 42, 70, 98, and 126 days after the quit date. All subjects received nicotine patches and were provided with behavioral treatment to maintain abstinence. Subjects were paid up to \$500 for 6 months of abstinence and adherence to urine collections.

**Urine Collection and Analysis.** Urine was collected from the first morning void up until, but not including, the first morning void of the next day. It was collected in 3-liter amber plastic containers. The urine was stored at  $-20^{\circ}\text{C}$  until analysis. NNAL and NNAL-Gluc were quantified as described (10). Enantiomers of NNAL and diastereomers of NNAL-Gluc were analyzed by CSP-GC-TEA, as described previously (13), except that a normal-phase HPLC cleanup step was added after the reverse-phase HPLC step. The residue from the reverse-phase cleanup was dissolved in 0.2 ml of ethyl acetate containing 5% isopropanol. Ten  $\mu\text{l}$  of isopropanol containing 1  $\mu\text{g}$  of 3-(3-pyridyl)propanol, as a retention time marker, were added to each sample. HPLC purification was performed on a Luna silica column ( $250 \times 4.6$  mm; 5- $\mu\text{m}$  bead size; Phenomenex, Torrance, CA). Solvent A was chloroform, and solvent B was chloroform containing 20% isopropanol. The solvent program was 10% A in B for 40 min at a flow rate of 1 ml/min. Eluant was collected for 8 min after the end of the marker peak. The collected material was concentrated to dryness and analyzed by CSP-GC-TEA.

Creatinine was assayed by Fairview-University Medical Center Diagnostic Laboratories (Minneapolis, MN), using Vitros CREA slides.

**Blood Collection and Analysis.** Blood was collected in EDTA-containing Vacutainers, and plasma was separated. Plasma (4.5 ml) was worked up for NNAL and NNAL-Gluc analysis by partitioning, as for the urine analysis (10). One ng of 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (*iso*-NNAL) was used as internal standard. Prior to reverse-phase HPLC, the organic extracts were concentrated to dryness, dissolved in 0.5 ml of 0.1% trifluoroacetic acid, and extracted twice with  $\text{CHCl}_3$ . The trifluoroacetic acid layer was neutralized and further purified by reverse-phase HPLC as in the analysis of urine. The HPLC fraction was concentrated to dryness and silylated with 5  $\mu\text{l}$  of bis-

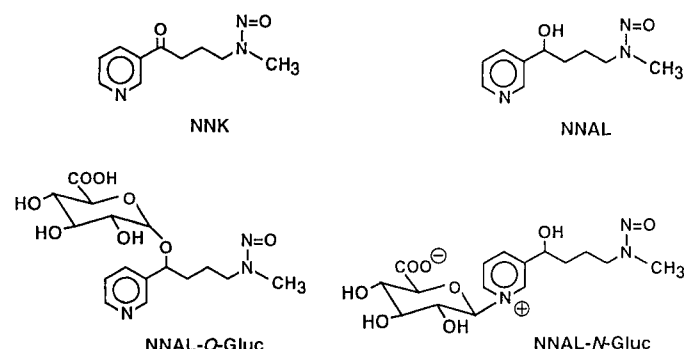


Fig. 1. Structures of NNK, NNAL, and NNAL-Gluc. The latter is a mixture of NNAL-O-Gluc and NNAL-N-Gluc.

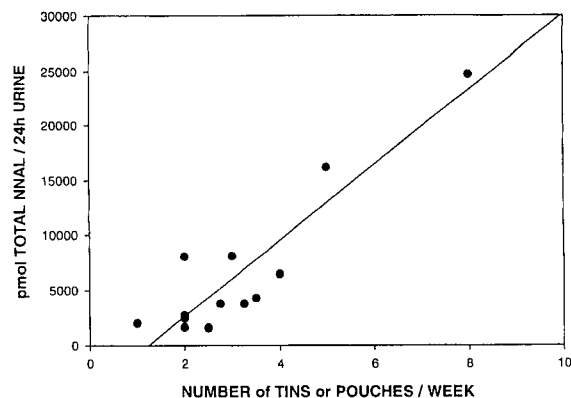


Fig. 2. Relationship between number of tins or pouches of smokeless tobacco used per week and NNAL plus NNAL-Gluc (TOTAL NNAL) in urine at baseline.

trimethylsilyltrifluoroacetamide containing 1% trimethylchlorosilane. Three  $\mu\text{l}$  were injected on the GC-TEA. Samples of  $\text{H}_2\text{O}$ , with and without 1.0 ng of added NNAL, were used as positive and negative controls. Two plasma samples from nontobacco users were used as additional negative controls.

**Pharmacokinetic Analysis.** Plasma levels of NNAL and NNAL-Gluc reached the limit of detection very quickly after cessation of smokeless tobacco use; therefore, urinary excretion rates of NNAL and NNAL-Gluc were used to determine the biological disposition of the two compounds. The urinary excretion rates (pmol/24 h) of NNAL and NNAL-Gluc were plotted as a function of time after quitting, with the rate at time zero being the average of the two baseline 24-h excretion rates taken within the 7-day period prior to cessation. The rate plots were generally fit to a biexponential equation with SAAM II, version 1.0.2, software (SAAM Institute, Seattle, WA):

$$\text{Urinary excretion rate (pmol/24 h)} = Ae^{-\alpha t} + Be^{-\beta t}$$

where  $\alpha$  and  $\beta$  are first-order rate constants with units of  $\text{days}^{-1}$ , and A and B are coefficients with units of pmol/24 h. In a few cases, a monoexponential equation was more appropriate and was used. The half-lives in the two phases were calculated by dividing 0.693 by either  $\alpha$  or  $\beta$ . The rate constants were compared between smokeless tobacco users and smokers with the use of the unpaired *t* test.

In seven subjects, serum samples were obtained and analyzed for NNAL and NNAL-Gluc; thus, renal clearance ( $\text{CL}_R$ ) rates for NNAL and NNAL-Gluc could be estimated:

$$\text{CL}_R = \frac{\text{Urinary excretion rate (pmol/24 h)}}{c}$$

where *c* is the serum concentration.

Table 1 Baseline data for NNAL and NNAL-Gluc in the urine of smokeless tobacco users

Subject	Product type <sup>a</sup>	pmol of NNAL			pmol of NNAL-Gluc			NNAL-Gluc/NNAL
		per ml of urine	per mg of creatinine	per 24-h urine	per ml of urine	per mg of creatinine	per 24-h urine	
1	S	0.82	0.513	1,070	2.67	1.56	3,230	3.26
2	C	1.31	0.999	2,300	3.25	2.49	5,760	2.48
3	S	0.41	0.402	594	1.31	1.24	1,840	3.20
4	S	0.81	0.866	1,830	2.60	3.03	6,290	3.21
5	C	0.32	0.211	336	1.24	0.85	1,290	3.88
6	S	0.75	0.558	802	2.69	2.02	2,980	3.59
7	S	2.95	1.98	3,900	9.09	5.83	12,300	3.08
8	S	3.13	3.07	6,760	8.56	8.39	18,000	2.73
9	S	0.77	1.42	1,870	1.93	3.48	4,600	2.51
10	S	0.667	0.452	842	2.14	1.56	2,930	3.21
11	S	1.41	0.995	773	2.16	1.51	1,230	1.53
12	S	0.47	0.280	393	1.35	0.851	1,180	2.87
13	S	0.55	0.438	731	1.46	1.15	1,970	2.65
Mean $\pm$ SD		1.11 $\pm$ 0.915	0.937 $\pm$ 0.813	1,710 $\pm$ 1,810	3.11 $\pm$ 2.61	2.61 $\pm$ 2.21	4,890 $\pm$ 4,980	2.94 $\pm$ 0.59
Mean $\pm$ SD in smokers <sup>b</sup>		0.601 $\pm$ 0.366	0.857 $\pm$ 0.514	944 $\pm$ 517	1.35 $\pm$ 0.738	1.84 $\pm$ 0.879	2,200 $\pm$ 1,130	2.73 $\pm$ 1.93

<sup>a</sup> S, moist snuff; C, chewing tobacco.

<sup>b</sup> From Hecht *et al.* (10).

Table 2 NNAL and NNAL-Gluc in plasma of smokeless tobacco users (pmol/ml of plasma)<sup>a</sup>

Subject	NNAL			NNAL-Gluc		
	Baseline	2 days post-quit	7 days post-quit	Baseline	2 days post-quit	7 days post-quit
7	0.178	0.152	0.022	0.100	0.090	Not done
8	0.189	0.206	0.023	0.189	0.167	Not done
9	0.202	0.143	0.008	0.136	0.081	Not done
10	0.348	0.203	ND <sup>b</sup>	0.104	0.100	ND
11	0.170	0.154	ND	0.177	0.057	ND
12	0.175	0.133	ND	0.114	0.087	ND
13	0.203	Not done	ND	0.073	0.037	ND
Mean $\pm$ SD	0.209 $\pm$ 0.063	0.165 $\pm$ 0.031	0.008 $\pm$ 0.009	0.127 $\pm$ 0.043	0.088 $\pm$ 0.041	

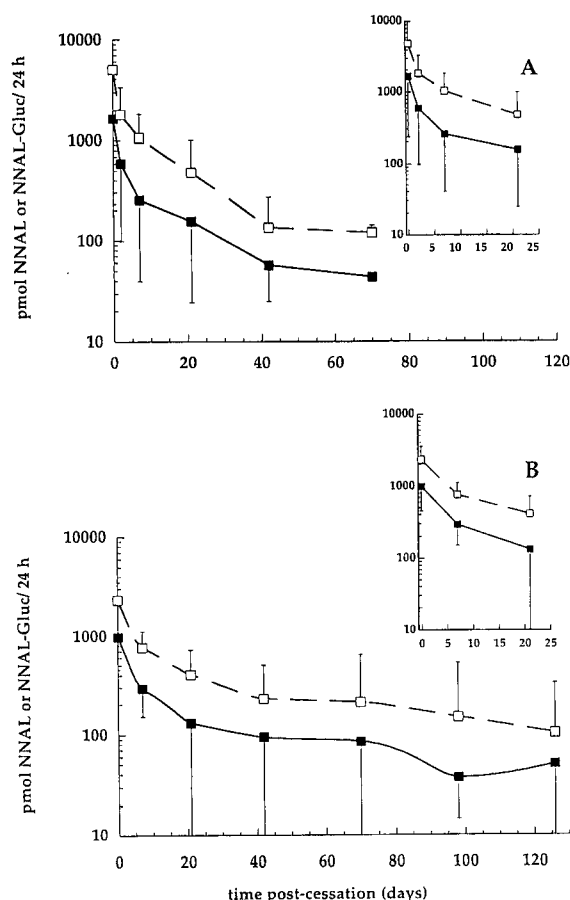
<sup>a</sup> All subjects were snuff dippers.<sup>b</sup> ND, not detected (detection limit, 0.01 pmol/ml).

Fig. 3. Mean urinary excretion rates (pmol/24 h) of NNAL (■) and NNAL-Gluc (□) as a function of time after cessation of smokeless tobacco use (A) or smoking (B). Insets show the data for the first 20 days.

## RESULTS

Thirteen subjects, all male nonsmokers (12 white, 1 black; age range, 27–61 years; mean  $\pm$  SD, 39.5  $\pm$  10.7 years) took part in the study. There were 11 snuff dippers and 2 tobacco chewers. The snuff dippers used 1–8 tins of smokeless tobacco per week (mean  $\pm$  SD, 3.4  $\pm$  1.9 tins per week). Ten of the 11 dippers used one of the two most popular brands on the market. The tobacco chewers used two pouches per week. One subject (subject 8) abstained only through day 21. His values are included in the baseline data (Table 1) and the plasma data (Table 2) but not in analyses of the urinary persistence studies.

Baseline urine data are summarized in Table 1. The mean levels of NNAL and NNAL-Gluc in the 24-h urine samples were 1710  $\pm$  1810 and 4890  $\pm$  4980 pmol, respectively, with ranges of 336–6760 pmol

for NNAL and 1,180–18,000 pmol for NNAL-Gluc. Urinary levels of NNAL plus NNAL-Gluc increased with increasing numbers of tins or pouches of smokeless tobacco used per week (Fig. 2;  $r = 0.91$ ;  $P < 0.01$ ). Mean baseline levels of NNAL plus NNAL-Gluc were somewhat higher in this study than in our previous study of smokers (10), but the differences were not significant. Ratios of NNAL-Gluc to NNAL were similar to those for smokeless tobacco users and smokers.

Plasma levels of NNAL and NNAL-Gluc were quantified in seven of the subjects at baseline and after 2 days of cessation, but were mainly nondetectable after 7 days of cessation (Table 2). Mean levels of NNAL and NNAL-Gluc at baseline were 0.209  $\pm$  0.063 and 0.127  $\pm$  0.043 pmol/ml, respectively. The mean NNAL level was 18.8% of the urinary amount, whereas the corresponding value for NNAL-Gluc was 4.1%. In our previous study, we measured NNAL in the plasma of four smokers, with a mean value of 0.063  $\pm$  0.049 pmol/ml (10).

Of the 13 subjects, 11 had adequate data for pharmacokinetic evaluation. The urine data from the present study and the previous one with smokers were fit to a biexponential equation (Fig. 3). In a few cases ( $n = 3$  for NNAL and  $n = 2$  for NNAL-Gluc), a monoexponential fit was more appropriate. The distribution phase of NNAL was significantly shorter in this study than in the smoking cessation study (Table 3; Fig. 3, insets). There was no significant difference in the terminal rate constants ( $\beta$ ) between the two studies. Because NNAL-Gluc is a formation rate-limited metabolite of NNAL, the apparent pharmacokinetic parameters mimic those of NNAL, as indicated in Table 3. From the terminal half-life data, it can be estimated that >4 months are required for NNAL to be eliminated from the body after cessation of tobacco use. Renal clearance data for NNAL and NNAL-Gluc are also presented in Table 3. Renal clearances were also plotted against the number of tins of smokeless tobacco used per week prior to cessation (Fig. 4).

Table 3 Pharmacokinetics of NNAL and NNAL-Gluc

	Smokers	Smokeless tobacco users	$P^a$
$t_{1/2\alpha}$ (days)			
NNAL	3.35 $\pm$ 1.86 ( $n = 22$ )	1.32 $\pm$ 0.85 ( $n = 9$ )	
NNAL-Gluc	3.89 $\pm$ 2.43 ( $n = 23$ )	1.53 $\pm$ 1.22 ( $n = 10$ )	
$\alpha$ (days <sup>-1</sup> )			
NNAL	0.257 $\pm$ 0.116 ( $n = 22$ )	0.71 $\pm$ 0.37 ( $n = 9$ )	<0.0001
NNAL-Gluc	0.260 $\pm$ 0.180 ( $n = 23$ )	0.72 $\pm$ 0.44 ( $n = 10$ )	0.0001
$t_{1/2\beta}$ (days)			
NNAL	45.2 $\pm$ 26.9 ( $n = 22$ )	26.3 $\pm$ 16.7 ( $n = 10$ )	
NNAL-Gluc	39.6 $\pm$ 26.0 ( $n = 26$ )	26.1 $\pm$ 15.1 ( $n = 10$ )	
$\beta$ (days <sup>-1</sup> )			
NNAL	0.024 $\pm$ 0.018 ( $n = 22$ )	0.033 $\pm$ 0.014 ( $n = 10$ )	0.148
NNAL-Gluc	0.025 $\pm$ 0.020 ( $n = 26$ )	0.035 $\pm$ 0.019 ( $n = 10$ )	0.192
Renal clearance (ml/min)			
NNAL	9.8 $\pm$ 8.0 ( $n = 3$ )	4.8 $\pm$ 4.1 ( $n = 7$ )	
NNAL-Gluc	ND <sup>b</sup>	25.2 $\pm$ 21.2 ( $n = 7$ )	

<sup>a</sup> Comparing smokers and smokeless tobacco users.<sup>b</sup> ND, not done.

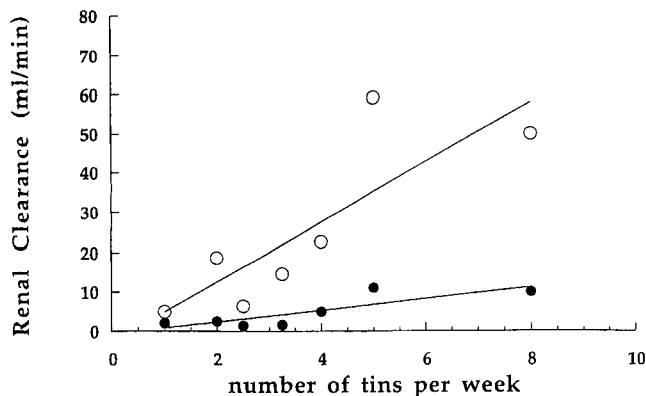


Fig. 4. Relationship between number of tins or pouches of smokeless tobacco used per week and renal clearance of NNAL (●) and NNAL-Gluc (○).

CSP-GC-TEA chromatograms of the NNAL and NNAL-Gluc fractions from the urine of a smokeless tobacco user, at baseline and after 7 days of cessation, are shown in Fig. 5. Similar chromatograms were obtained when the corresponding urine samples from smokers were analyzed. This analysis was carried out on urine samples from five smokeless tobacco users and five smokers. The results are summarized in Fig. 6 and Table 4. At day 7, the levels of NNAL or NNAL-Gluc in some subjects were too low to quantify by CSP-GC-TEA, which is less sensitive than the conventional method. However, in all subjects for whom data were available, the ratio (S)-NNAL:(R)-NNAL or (S)-NNAL-Gluc:(R)-NNAL-Gluc was higher at day 7 than at baseline. All differences between day 7 and baseline were significant in cases where sufficient data were available (Table 4).

## DISCUSSION

Two observations in this study may have considerable implications with regard to our understanding of NNK metabolism and pharmacokinetics in people who use tobacco products. The first observation is that the distribution half-lives of NNAL and NNAL-Gluc were significantly shorter in smokeless tobacco users than in smokers. The second observation is the ratios (S)-NNAL:(R)-NNAL and (S)-NNAL-Gluc:(R)-NNAL-Gluc increased in both smokers and snuff dippers after cessation.

The shorter distribution half-lives of NNAL and NNAL-Gluc in smokeless tobacco users than in smokers are consistent with our hypothesis that NNK and/or NNAL are sequestered in the lung, possibly in  $\beta_2$ -adrenergic receptors, as discussed below. We expect that the initial dose of NNK to the lung will be greater in smokers than in snuff dippers and that the lung may act in part as a first-pass uptake tissue for NNK in smokers. Therefore, the apparently longer initial distribution phases of NNAL and NNAL-Gluc in smokers than in smokeless tobacco users imply that the lung is one of the sequestering tissues. However, it should be noted that more complete data were collected in the distribution phase of the smokeless tobacco users (an additional urine sample at 2 days postcessation) than of the smokers (Fig. 3A, inset, versus Fig. 3B, inset). This additional data point could have influenced the estimate of the distribution rate constant in the smokeless tobacco users.

The increases in the (S)-NNAL:(R)-NNAL and (S)-NNAL-Gluc:(R)-NNAL-Gluc ratios after cessation were striking and consistent. We propose that these increases result from the presence in the body of receptor(s) that preferentially bind (S)-NNAL. As (S)-NNAL is slowly released from the receptor, it is converted to (S)-NNAL-Gluc. The receptor could be a  $\beta_2$ -adrenergic receptor. The natural substrate

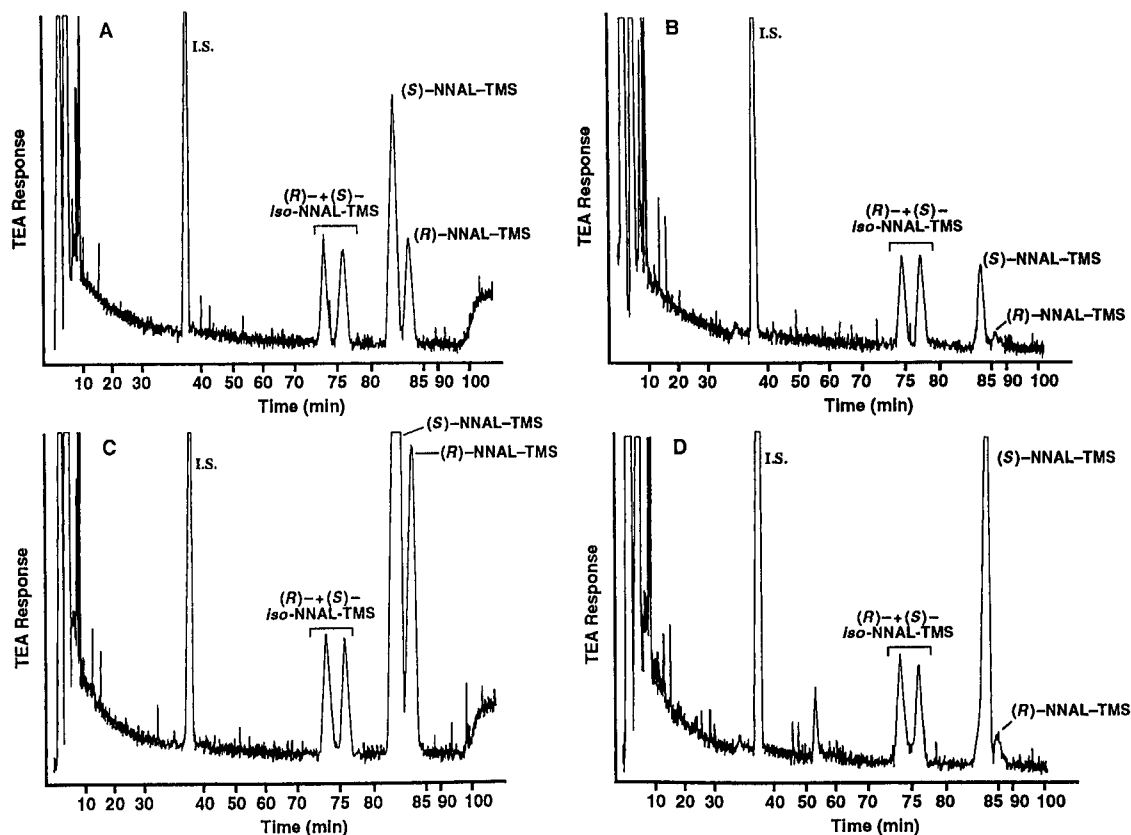
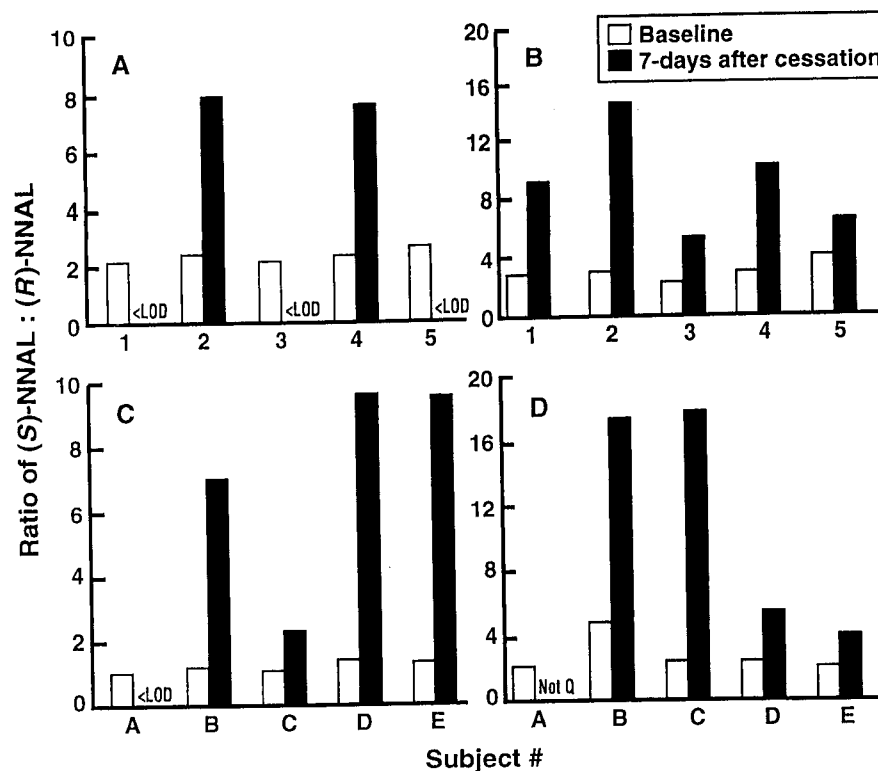


Fig. 5. CSP-GC-TEA chromatograms of the NNAL fraction (A and B) and NNAL-Gluc fraction (C and D) at baseline (A and C) and 7 days after cessation of smokeless tobacco use (B and D). I.S., injection standard (*N*-nitrosopentyl-3-picolyamine).

Fig. 6. Ratios (*S*)-NNAL:(*R*)-NNAL (A and C) and (*S*)-NNAL-Gluc:(*R*)-NNAL-Gluc (B and D) in smokeless tobacco users (A and B) and smokers (C and D) at baseline (open columns) and 7 days after cessation (filled columns). LOD, limit of detection (0.4 ng/sample); Not Q, not quantified



for the  $\beta_2$ -adrenergic receptor is (*R*)-epinephrine, which is not structurally dissimilar to (*S*)-NNAL (Fig. 7; Ref. 14).  $\beta_2$ -Adrenergic receptors are found in smooth muscle (vascular, bronchial, gastrointestinal, and genitourinary) and in skeletal muscle (14). Schuller *et al.* (15) have already shown that NNK is a  $\beta$ -adrenergic agonist and stimulates DNA synthesis in cell lines via receptor-mediated release of arachidonic acid. However, they did not investigate NNAL or its enantiomers. Increased release of arachidonic acid could lead to increased production of prostaglandin  $E_2$ , which has been implicated in several studies as a key intermediate in NNK-induced lung tumorigenesis (16, 17). The present data are also supported by our previous work in rats, indicating that (*S*)-NNAL is stereoselectively sequestered in the lung of rats at prolonged times after dosing.<sup>4</sup> These data are particularly interesting in view of the fact that (*S*)-NNAL is the more carcinogenic of the NNAL enantiomers (18).

In this study, we present data on both NNAL and NNAL-Gluc in human blood. Together with our previous investigation of smokers (10), the results presented here establish the feasibility of quantifying these metabolites in human plasma. Previous studies have reported amounts of the nicotine metabolite cotinine in both blood and urine (19–21). Cotinine in plasma was ~20% of the amount in urine, which can be compared with the 18.8% value for NNAL obtained in this study. These data indicate similarities between the disposition of

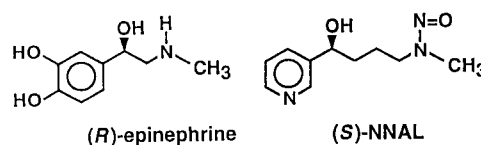


Fig. 7. Structures of (*R*)-epinephrine and (*S*)-NNAL.

nicotine and NNK, as indicated by their metabolites, cotinine and NNAL. Plasma NNAL and NNAL-Gluc may be useful biomarkers for NNK uptake in future molecular epidemiological studies because blood samples are more frequently banked than urine samples. An added bonus of the quantitation of NNAL and NNAL-Gluc in plasma is the ability to determine the renal clearances of the two compounds. The renal clearance of NNAL-Gluc is 5-fold greater than that of NNAL, as would be expected for a glucuronide metabolite. Because the glomerular filtration rate in humans is ~120 ml/min and because NNAL does not appear to be bound to plasma proteins (10), both NNAL and NNAL-Gluc appear to be reabsorbed in the renal tubules, although NNAL-Gluc is less so.

We observed a correlation between baseline total NNAL plus NNAL-Gluc in urine and number of tins or pouches per week of smokeless tobacco consumed (Fig. 2). These data provide further support for the concept that NNAL plus NNAL-Gluc is a biomarker of NNK uptake from tobacco. In our previous study of smokeless tobacco users, NNAL plus NNAL-Gluc correlated with cotinine in tobacco chewers but not in snuff dippers (22). We have noted a correlation between NNAL plus NNAL-Gluc and urinary cotinine plus cotinine-*N*-glucuronide in smokers (9). Thus, cotinine is a biomarker of uptake of the addictive compound nicotine, whereas NNAL plus NNAL-Gluc is a biomarker of uptake of the carcinogen NNK. There was also an apparent correlation between the renal clearances of NNAL and NNAL-Gluc and the number of tins of tobacco used per week prior to cessation (Fig. 4). This is reminiscent of the well-known relationship of nicotine levels in the body and

Table 4 (*S*):(*R*) ratios for NNAL and NNAL-Gluc in smokeless tobacco users and smokers at baseline and 7 days after cessation

	( <i>S</i> ):( <i>R</i> ) ratios <sup>a</sup>		<i>p</i> <sup>b</sup>
	Baseline	7 days	
Smokeless tobacco users			
NNAL	2.12 ± 0.51	7.80 ± 0.22 ( <i>n</i> = 2)	0.006
NNAL-Gluc	2.94 ± 0.57	9.10 ± 3.67	
Smokers			
NNAL	1.23 ± 0.15	7.07 ± 3.41 ( <i>n</i> = 4)	0.006
NNAL-Gluc	2.84 ± 1.17	11.19 ± 7.50 ( <i>n</i> = 4)	0.04

<sup>a</sup> Mean ± SD, *n* = 5, unless noted otherwise.

<sup>b</sup> Comparing baseline and 7-day values.

self-regulation of nicotine intake for smokers (23). If nicotine is eliminated from the body more quickly in some smokers, they may have an increased nicotine intake to compensate for the increased clearance (24). When the urine is acidified, leading to a large increase in nicotine renal clearance, nicotine intake from cigarette smoking significantly increases (25). Conversely, when nicotine metabolism by cytochrome P450 2A6 was inhibited by methoxsalen, plasma nicotine levels increased and the number of cigarettes smoked decreased (26). The apparent relationship between NNAL and NNAL-Gluc renal clearances and smokeless tobacco intake is likely a reflection of the relationship between nicotine pharmacokinetics and the self-regulation of nicotine intake by smokeless tobacco users.

The baseline data for NNAL and NNAL-Gluc measured in this study are similar to those of our previous study of smokeless tobacco users from Ohio (22). In that study of 39 dippers and chewers, mean levels of NNAL and NNAL-Gluc in urine were  $0.92 \pm 1.59$  and  $3.47 \pm 5.86$  pmol/mg of creatinine, respectively, compared with the corresponding levels of  $0.937 \pm 0.813$  pmol/mg of creatinine for NNAL and  $2.61 \pm 2.21$  pmol/mg of creatinine for NNAL-Gluc in this study. These values are not significantly different and reflect consistent NNK uptake among smokeless tobacco users.

NNK is a systemic carcinogen in rats, inducing mainly lung tumors independent of the route of administration (1). Lung adenoma and adenocarcinoma have been observed in rats treated with NNK by s.c. injection, by administration in the drinking water, by swabbing in the oral cavity, by intragastric gavage, and by intravesicular administration. In humans, the dose of NNK to the lung is likely to be less in smokeless tobacco users than in smokers, and there are differences in the pharmacokinetics, as noted above. Nevertheless, overall uptake of NNK in smokeless tobacco users and smokers is similar, based on urinary levels of NNAL plus NNAL-Gluc (Table 1). When this is considered together with the animal carcinogenicity data cited above, it raises the realistic possibility that smokeless tobacco users could be at risk for lung cancer. This should be investigated in epidemiological studies. Recent epidemiological studies have demonstrated increased risk for lung cancer in cigar smokers who did not inhale, consistent with the presence of a systemic carcinogen in cigar smoke (27). Levels of NNK in cigars have been reported to be 1200 ng/cigar (27).

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