

Quantitation of Urinary Metabolites of a Tobacco-specific Lung Carcinogen after Smoking Cessation¹

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ABSTRACT

We quantified urinary levels of two metabolites of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in people who had stopped smoking: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its *O*-glucuronide, 4-[(methylnitrosamino)-1-(3-pyridyl)but-1-yl]- β -*O*-D-glucosiduronic acid (NNAL-Gluc). Twenty-seven people completed the study. Thirteen used the nicotine patch starting at the quit date, whereas the others used no patch. Two 24-h urine samples were collected on 2 consecutive days before smoking cessation; blood was also obtained. Beginning at their quit date, subjects provided 24-h urine samples on days 7, 21, 42, 70, 98, and 126, and some subjects also provided samples at later times. The urine was analyzed for NNAL, NNAL-Gluc, nicotine plus nicotine-*N*-glucuronide, and cotinine plus cotinine-*N*-glucuronide. Some blood samples were also analyzed for NNAL. The decline of urinary NNAL and NNAL-Gluc after smoking cessation was much slower than expected. This was clearly demonstrated by comparison with cotinine and nicotine levels in urine. One week after smoking cessation, 34.5% of baseline NNAL plus NNAL-Gluc was detected in urine, whereas the corresponding values for cotinine and nicotine were 1.1 and 0.5%, respectively. Even 6 weeks after cessation, 7.6% of the original levels of NNAL plus NNAL-Gluc remained. In some subjects, NNAL plus NNAL-Gluc were detected 281 days after cessation. The distribution half-life for NNAL and NNAL-Gluc was 3–4 days, whereas the elimination half-life was 40–45 days. Total body clearance of NNAL was estimated to be 61.4 ± 35.4 ml/min, and volume of distribution in the β -phase was estimated to be 3800 ± 2100 liters, indicating substantial distribution into the tissues. Parallel studies in rats treated chronically or acutely with NNK in the drinking water support the conclusion that NNAL has a large volume of distribution. There was no effect of the nicotine patch on levels of NNAL plus NNAL-Gluc, indicating that NNK is not formed endogenously from nicotine. The results of this study demonstrate that NNAL and NNAL-Gluc are slowly cleared from the body after smoking cessation, indicating the presence of a high-affinity compartment where NNK, NNAL, and/or NNAL-Gluc are retained or sequestered and slowly released.

INTRODUCTION

In this study, we quantified two metabolites, NNAL³ and NNAL-Gluc, of the tobacco-specific lung carcinogen NNK in the urine of people who had stopped smoking for 4 months. NNK is believed to play a significant role as a cause of lung cancer in smokers (1–3). NNK is a potent pulmonary carcinogen in rodents, inducing adenocarcinoma of the lung, independent of the route of administration (1). The lowest total doses of NNK that can produce lung tumors in rats are similar to the lifetime total dose of NNK in a smoker (1). Biochemical studies indicate that the same routes of metabolic acti-

vation and detoxification of NNK exist in rodents and humans, although there are quantitative differences (1). Major pathways of NNK metabolism are summarized in Fig. 1 (1). In rodents and humans, NNK is rapidly metabolized to its carbonyl reduction product NNAL, which is also a pulmonary carcinogen in rats and mice. NNAL is *O*-glucuronidated to give NNAL-Gluc, which is excreted in urine. Urinary NNAL and NNAL-Gluc are excellent biomarkers for NNK uptake in smokers and in people exposed to environmental tobacco smoke (1). NNK itself is not detected in urine due to its extensive metabolism. Both NNAL and NNK undergo metabolic hydroxylation at the carbons adjacent to the nitrosamino nitrogen. These cytochrome P450-catalyzed α -hydroxylation reactions generate intermediates that react with DNA, causing permanent mutations associated with tumor induction (1).

Little is known about the excretion of carcinogens and other tobacco smoke constituents after smoking cessation. Two studies examined the decrease of 4-aminobiphenyl hemoglobin adducts after withdrawal from smoking; the decline was faster than predicted, based on the lifetime of the red cell (4, 5). Polycyclic aromatic hydrocarbon-DNA adducts in leukocytes, as determined by immunoassay, also decreased after smoking cessation (5). Other indicators of DNA damage, such as glycophorin A mutations or BPDE-DNA adduct antibodies, were unaffected by cessation (5, 6). Among urinary constituents related to smoking, only 8-oxodeoxyguanosine and cotinine appear to have been studied. 8-Oxodeoxyguanosine, a product of oxidative damage by cigarette smoke, decreased by 21% 4 weeks after cessation (7). Urinary cotinine, a major metabolite of nicotine, is reported to have a half-life of 16.5 h after cessation (8). NNK and NNAL are structurally related to nicotine and cotinine. On this basis alone, one would expect fairly rapid elimination of NNK metabolites after smoking cessation. However, our preliminary data suggested that this was not the case. Therefore, we investigated the persistence of NNAL and NNAL-Gluc in people who had quit smoking. As the results will show, they were far more persistent than either nicotine or cotinine, suggesting that NNK or NNAL was slowly eliminated from the body.

MATERIALS AND METHODS

Study Design. The study was approved by the University of Minnesota Research Subjects' Protection Programs Institutional Review Board Human Subjects Committee. The study design is summarized in Fig. 2. Smokers were recruited through newspaper advertisements and local press releases. Subjects were initially screened over the telephone. They must have smoked at least 15 cigarettes per day for the past year. Potential subjects were scheduled for a screening visit at the University of Minnesota Tobacco Research Programs clinic. At the screening visit, they filled out a questionnaire regarding their smoking history and completed a medical history form. Smoking status was confirmed by salivary cotinine and expired CO. Subjects were excluded if they had any current medical condition, used prescribed medications, or had a psychiatric diagnosis. Qualified subjects smoked normally for 7 days prior to their quit date. Two 24-h urine samples were collected on 2 consecutive days during this period and analyzed for nicotine, cotinine, NNAL, and NNAL-Gluc. Blood samples were also obtained. Subjects reported to the study clinic on the evening before their quit date. Data were collected on vital signs,

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³ The abbreviations used are: NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNAL-Gluc, 4-[(methylnitrosamino)-1-(3-pyridyl)but-1-yl]- β -*O*-D-glucosiduronic acid; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; GC-TEA, gas chromatography-nitrosamine-selective detection; TMS, trimethylsilyl ether.

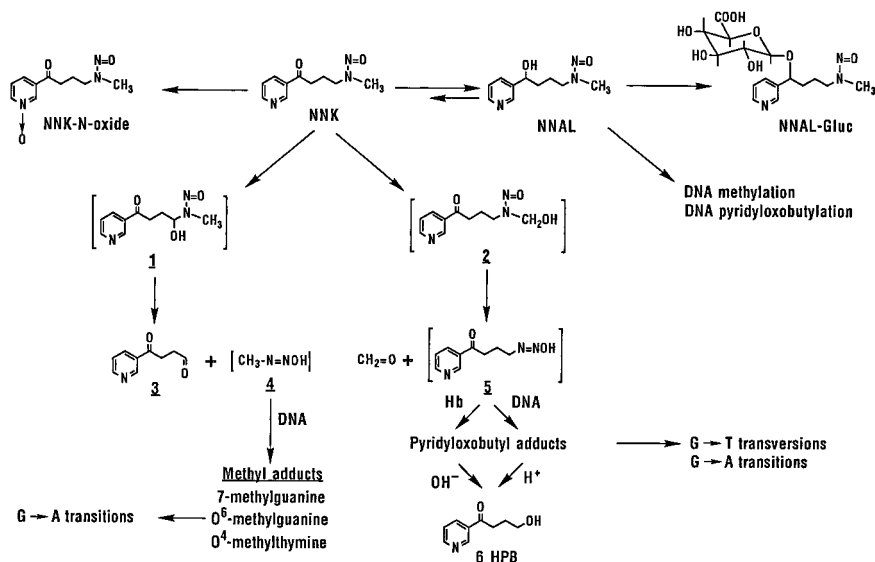


Fig. 1. Overview of NNK metabolism. Details have been presented previously (1).

concomitant medications, CO levels, and self-reported smoking status. They were randomly assigned to active nicotine patch (15-mg Nicotrol Nicotine Transdermal System), or no patch. Adjunct counseling consisted of group counseling by trained smoking cessation group counselors over the course of 7 weeks, with patch use for 6 of those weeks. Subjects attended biweekly sessions for 3 weeks and weekly sessions for 4 weeks. Follow up visits occurred at 4, 8, 12, and 26 weeks post-treatment. Beginning at the quit date, subjects visited the study center and provided 24 h urine at days 7, 21, 42, 70, 98, and 126 thereafter. Some subjects also provided samples 196, 211, and 281 days after quitting. The urine was analyzed for nicotine, cotinine, NNAL, NNAL-Gluc, and creatinine. At each visit, data were collected on expired CO, vital signs, concomitant medications, and self-reported smoking status.

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 adjusted to 0.1 N NaOH with 10N NaOH and stored at -20°C until analysis. NNAL and NNAL-Gluc were analyzed essentially as described (9), except that capillary GC-TEA and confirmation by gas chromatography-tandem mass spectrometry were used (10).

Nicotine and cotinine were analyzed by a method similar to those described previously (11, 12). $[\text{CD}_3]\text{Cotinine}$ and $[\text{CD}_3]\text{nicotine}$ (Sigma Chemical Co., St. Louis, MO; 500 ng of each for active smokers; 500 ng cotinine and 50 ng nicotine in quitters) were used as internal standards. They were added to urine (0.2 ml from active smokers and 1 ml from quitters). The volume of urine from the active smokers was adjusted to 1 ml. It was mixed with 1 ml of 50% aqueous K_2CO_3 and then extracted once with 2 ml of CH_2Cl_2 . The CH_2Cl_2 layer was separated and mixed with 200 μl of CH_3OH . This solution was concentrated under a gentle stream of N_2 to a total volume of 100–200 μl of CH_3OH and then analyzed by gas chromatography-mass spectrometry-selected ion monitoring (model 6890 gas chromatograph equipped with an autosampler

and model 5973 Mass Selective Detector; Hewlett Packard, Palo Alto, CA). The GC was equipped with a Merlin septum and an HP-5 fused silica capillary column (30 m, 0.25 mm inner diameter, 0.25- μm film thickness). The injection port temperature was 250°C , and the injection mode was splitless. Deactivated injection port liners were used as described (11). The oven temperature was 70°C for 0.5 min and then increased to 180°C at $10^{\circ}\text{C}/\text{min}$, held for 1 min, increased to 275°C at $50^{\circ}\text{C}/\text{min}$, held for 5 min, and returned to initial conditions. The carrier gas was He at a flow rate of 1 ml/min. The mass spectrometry transfer line was heated at 300°C . From 0 to 10 min, we monitored m/z 84 $[\text{C}_4\text{H}_7\text{NCH}_3]^+$, 87 $[\text{C}_4\text{H}_7\text{NCD}_3]^+$, 162 $[\text{M}^+, \text{nicotine}]$, and 165 $[\text{M}^+, [\text{CD}_3]\text{nicotine}]$; after 10 min, we monitored m/z 98 $[\text{C}_4\text{H}_5\text{ONCH}_3]^+$, 101 $[\text{C}_4\text{H}_5\text{ONCD}_3]^+$, 176 $[\text{M}^+, \text{cotinine}]$, and 179 $[\text{M}^+, [\text{CD}_3]\text{cotinine}]$. The retention time of nicotine was 8.37 min, and that of cotinine was 12.78 min. The internal standards eluted 0.02 min before the analytes. Because the samples were stored in base, nicotine-*N*-glucuronide, and cotinine-*N*-glucuronide were hydrolyzed to free nicotine and cotinine by heating at 80°C for 1 h. The nicotine values include nicotine and nicotine-*N*-glucuronide, and the cotinine values include cotinine and cotinine-*N*-glucuronide.

Blood Collection and Analysis. Blood was collected in EDTA-containing vacutainers. RBCs and plasma were separated, the RBCs were washed three times with two volumes of 0.9% saline, and both fractions were stored at -80°C until analysis. The washed RBC pellet from 14 ml of blood was lysed with H_2O with vortexed. The mixture was extracted twice with hexane. The hexane extracts were discarded. The aqueous phase was extracted three times with CH_2Cl_2 , and the combined extracts were analyzed for unconjugated NNAL and NNK as described for urine. The aqueous phase was treated with 0.1 N NaOH, with sonication for 1 h at room temperature. After neutralization, it was extracted three times with equal volumes of CH_2Cl_2 and then analyzed for NNAL and NNK as above. The aqueous phase was acidified to a concentration of 0.1 N HCl. It was sonicated for 1 h at room temperature, neutralized, and extracted three times with CH_2Cl_2 , and the extracts analyzed for NNAL and NNK. Plasma from the same blood sample was treated and analyzed the same way as RBCs.

Other Analyses. CO was determined with a Medical Gas Monitor, Bedford Scientific, Ltd. (Kent, United Kingdom). Creatinine was assayed by Fairview-University Medical Center Diagnostic Laboratories (Minneapolis, MN) using Vitros CREA slides.

Pharmacokinetic Analyses. The urinary excretion rates (dX_e/dt , pmol per 24 h) of NNAL and NNAL-Gluc were plotted separately as a function of time (days postquitting) with the rate at time zero being the steady-state urinary excretion rate on the last day of smoking. The rate-time plots appeared to be biphasic and were fit to a bi-exponential model with the use of KaleidaGraph (Version 3.08d; Synergy Software):

$$dX_e/dt = Ze^{-\alpha t} + Ye^{-\beta t}$$

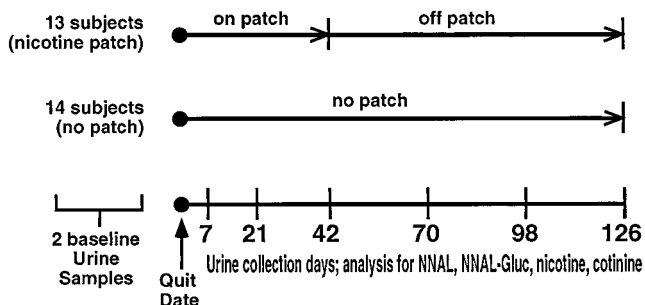


Fig. 2. Design for the study of persistence of urinary metabolites of NNK after smoking cessation.

In three smokers, both 24-h urine collections and blood samples that had been collected at approximately the midpoint of the urine collection were available. Assuming steady-state conditions, from these data it was possible to obtain estimates of the renal clearance of NNAL (CL_r) and its total body clearance (CL):

$$CL_r = \frac{dXe/dt}{C_{ss}}$$

$$CL = \frac{D}{C_{ss}}$$

where *D* is the "daily dose" of NNAL and *C_{ss}* is the steady-state concentration of NNAL in the blood. The daily dose of NNAL was estimated under the following assumptions: (a) the average amount of NNK delivered to a smoker per cigarette is 200 ng; (b) 50% of NNK is metabolized to NNAL; and (c) the dose of NNAL can be calculated from the average daily self-reported number of cigarettes smoked. Although we recognize that these assumptions may not be entirely valid, they were nevertheless useful in obtaining preliminary estimates of pharmacokinetic parameters.

The volume of distribution in the β-phase (*V_β*) was also calculated:

$$V_{\beta} = \frac{CL}{\beta}$$

In the acute and chronic rat studies described below, NNAL and NNAL-Gluc excretion rates appeared to follow biexponential pharmacokinetics. Only NNAL-Gluc from the chronic studies could actually be fit to a biexponential equation. Therefore, only the rate constants from the terminal phase were determined in rats.

Rat Study. Six male F-344 rats weighing about 250 g were obtained from Charles River Breeding Laboratories (Kingston, NY). They were maintained on NIH-07 diet. Two groups of three rats each were used for chronic and acute studies. In the chronically treated group, three rats were given NNK in the drinking water at 2 ppm for 14 days. After 2 weeks, they were placed in metabolism cages. NNK was continued in the drinking water, and 24-h urine was collected from the rats. They were then returned to conventional cages for an additional 2 weeks of NNK treatment. NNK administration ceased after a total of 28 days. The rats were then placed in metabolism cages, and their urine

was collected for 5 days. In the acutely treated group, three rats were given NNK in the drinking water at 2 ppm for 2 days. They were then transferred to metabolism cages and urine was collected for the next 5 days. Urine from both groups of rats was analyzed for NNAL and NNAL-Gluc as above. For all rats, mean daily NNK dose was estimated to be 0.22 μmol (13).

Statistical Analysis. A paired *t* test was used to compare α and β from NNAL and NNAL-Gluc. ANOVA for repeated measurements was used to compare the elimination of NNAL and NNAL-Gluc in subjects who did or did not use the nicotine patch.

RESULTS

Forty-four people enrolled in the study. One did not complete it due to a diagnosis of lung cancer, whereas 14 others could not abstain from cigarettes for 126 days. Of the 29 people who completed the program, two were excluded because cotinine data indicated that they smoked during the cessation period. The 27 people for whom we are reporting data smoked an average of 23.7 ± 6.9 cigarettes/day (range, 15–40 cigarettes/day) for an average of 21.1 ± 11.3 years (range, 1–42 years). There were 13 females. The average age was 43.9 ± 11.1 years (range, 24–64 years) and the racial makeup was 96% Caucasian and 4% African-American. Measurements of cotinine and exhaled CO indicated their compliance with the cessation program.

Baseline data are summarized in Table 1. The mean level ± SD and range of NNAL per 24 h were 944 ± 517 pmol and 180–2080 pmol, respectively. The corresponding values for NNAL-Gluc per 24 h were 2200 ± 1130 pmol and 280–4970 pmol. NNAL plus NNAL-Gluc correlated with cotinine (*R* = 0.43; *P* = 0.0079) and nicotine (*R* = 0.44; *P* = 0.006). These data are consistent with previous observations (9, 10).

Typical GC-TEA traces of the NNAL-Gluc fraction from the urine of one individual over the course of the study are illustrated in Fig. 3. In this analysis, NNAL is released from NNAL-Gluc by hydrolysis with β-glucuronidase. After extraction and purification, it is silylated to give NNAL-TMS, which is analyzed by GC-TEA. The NNAL-

Table 1 Baseline data for NNAL, NNAL-Gluc, cotinine, and nicotine in urine^a

Subject	pmol of NNAL			pmol of NNAL-Gluc			Cotinine (nmol/ml urine)	Nicotine (nmol/ml urine)
	Per ml	Per mg of creatinine	Per 24 h	Per ml	Per mg of creatinine	Per 24 h		
1	0.285 ^b	0.548	684	0.728 ^b	1.40	1750	8.65	23.2
2	0.87	1.45	783	1.11	1.43	999	18.3 ^b	22.0 ^b
3	1.18	1.20	1000	1.95	2.10	1650	14.6	11.4
4	0.181 ^b	0.312	199	0.364 ^b	0.629	400	5.57	1.49
5	0.673	0.802	942	1.79	2.11	2500	11.6 ^b	9.23 ^b
6	0.525	0.491	197	0.747	0.647	280	11.0 ^b	2.93 ^b
7	0.096 ^b	0.089	180	1.09 ^b	1.01	2050	8.40	9.71
8	0.586	2.37	908	1.28	5.03	1980	9.25	9.86
9	0.746	0.863	1720	1.64	1.88	3690	10.3	12.3
10	0.419	1.78	712	0.876	3.00	1490	10.7 ^b	11.8 ^b
11	0.743	0.639	1190	2.52	2.14	4030	10.7 ^b	5.53 ^b
12	0.636	0.948	922	1.09	1.50	1580	13.4	13.0
13	0.905	0.929	1370	2.48	2.57	3760	15.9	12.4
14	0.265	0.946	410	0.792	2.22	1230	14.9 ^b	9.94 ^b
15	1.26	0.88	1820	3.43	2.40	4970	13.3	36.4
16	0.348 ^b	0.523	645	1.49 ^b	2.23	2750	14.3	16.1
17	0.201	0.724	1000	0.408	1.60	2040	10.3 ^b	7.29 ^b
18	0.589 ^b	1.14	1330	1.38 ^b	2.76	3090	7.60	8.11
19	0.367	0.489	1060	1.02	1.29	2950	21.4 ^b	14.3 ^b
20	0.109 ^b	0.373	295	0.538 ^b	1.83	1450	6.70 ^b	4.27 ^b
21	0.496	0.366	459	1.26	1.21	1170	36.3 ^b	30.8 ^b
22	0.915	0.469	1330	2.27	1.16	3290	19.1 ^b	17.9 ^b
23	0.308	0.671	1130	0.708	1.54	2590	6.59	16.4
24	1.18	1.79	1770	1.23	1.86	1840	23.8 ^b	15.6 ^b
25	0.356	0.714	748	0.589	0.942	1210	2.63	5.16
26	1.44	1.10	2080	2.07	1.59	3000	13.6 ^b	17.6 ^b
27	0.543 ^b	0.520	624	1.55 ^b	1.49	1780	14.0	23.6
Mean ± SD	0.601 ± 0.366	0.857 ± 0.514	944 ± 517	1.35 ± 0.738	1.84 ± 0.879	2200 ± 1130	13.1 ± 6.71	13.6 ± 8.22

^a Data are means of two baseline day values, except where noted.

^b Single value.

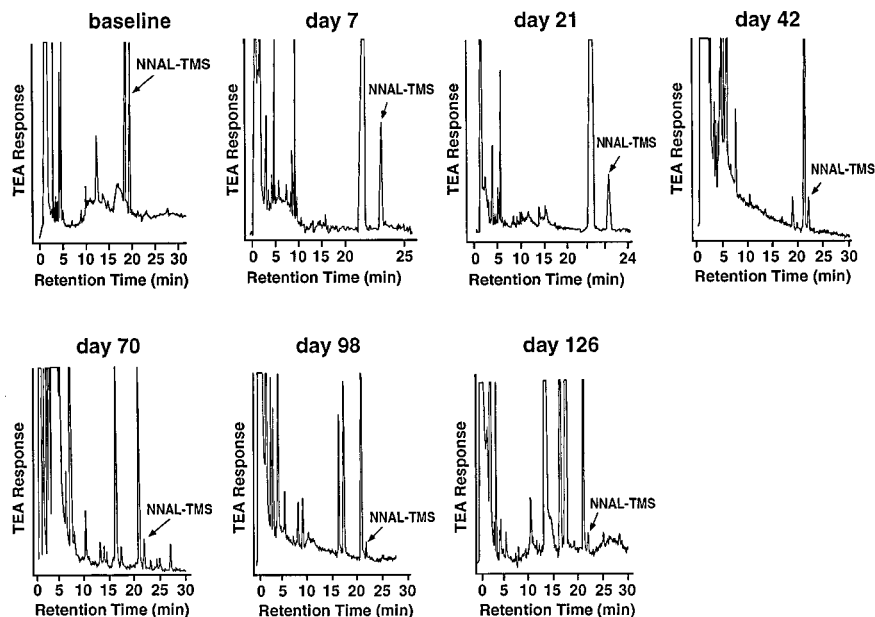


Fig. 3. GC-TEA traces of the NNAL-Gluc fraction from the urine of one subject at baseline and at various days after smoking cessation.

TMS peaks at the later time points were quite small. For representative samples, the identities of these peaks as NNAL-TMS were confirmed by gas chromatography-tandem mass spectrometry.

Fig. 4 depicts the mean excretion rate data for NNAL and NNAL-Gluc in the 27 subjects. Of the 27 individuals, 22 showed clear biexponential pharmacokinetics for NNAL, and 23 showed biexponential pharmacokinetics for NNAL-Gluc. One subject could not be fit to any pharmacokinetic model due to variability in his data, and the excretion rate data in the others appeared to be monoexponential. In general, there was a relatively rapid initial decline in excretion rates followed by a slower terminal phase. Although the NNAL-Gluc excretion rate was significantly higher in all urine samples, the excretion rate-time profiles for NNAL and NNAL-Gluc were parallel, indicating that NNAL-Gluc was formation rate limited in its elimination. Indeed, there was no significant difference in either the distribution rate constant (α) or elimination rate constants (β) for the two compounds (Table 2). The distribution half-life for the two compounds was 3–4 days, and the elimination half-life was 40–45 days, indicating prolonged retention of NNAL and NNAL-Gluc after cessation of smoking.

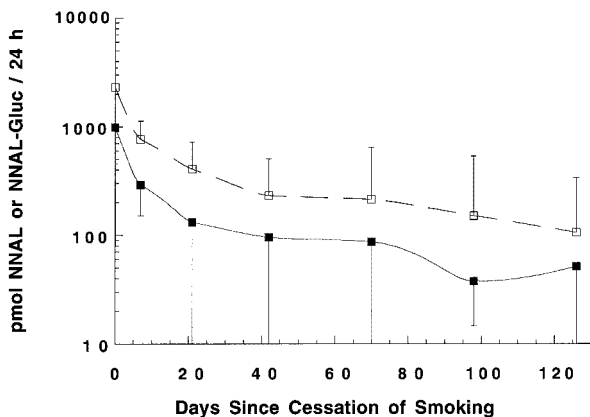


Fig. 4. Mean urinary excretion rates (pmol/24 h) of NNAL (■) and NNAL-Gluc (□) in 27 subjects as a function of time after smoking cessation. Note that excretion rates are presented on a semilogarithmic scale, which exaggerates the appearance of the error bars at the lower range of the Y axis. Data points, means; bars, SD.

Table 2 Pharmacokinetics of NNAL and NNAL-Gluc in humans^a

	NNAL	NNAL-Gluc
α (day ⁻¹)	0.257 ± 0.116 (n = 22)	0.260 ± 0.180 (n = 23)
$t_{1/2\alpha}$ (days)	3.35 ± 1.86 (n = 22)	3.89 ± 2.43 (n = 23)
β (day ⁻¹)	0.0235 ± 0.0182 (n = 22)	0.025 ± 0.0201 (n = 26)
$t_{1/2\beta}$ (days)	45.2 ± 26.9 (n = 22)	39.6 ± 26.0 (n = 26)

^aData (mean ± SD) were obtained from the biexponential fit of excretion rate data. There was no significant difference ($P < 0.05$) in the distribution rate constants or elimination rate constants for NNAL or NNAL-Gluc.

Two subjects were recalled 196 and 211 days after cessation; NNAL and NNAL-Gluc were not detected in one sample and totaled 0.071 pmol/ml in the other. Five subjects were recalled 281 days after cessation. NNAL and NNAL-Gluc were not detected in three of the urine samples, whereas the total amounts in the other two were 0.025 and 0.029 pmol/ml. The mean background level of NNAL plus NNAL-Gluc in urine samples from nonsmokers, which were analyzed at the same time as the study samples, was 0.016 ± 0.029 pmol/ml (n = 21). These data suggest that NNAL plus NNAL-Gluc may persist for longer than 135 days in some individuals.

Individual values of NNAL plus NNAL-Gluc per 24 h during the cessation period were compared to the mean baseline values for each subject. The baseline values were set as 100%, and the percentage of baseline for each of the 14 people who did not use the nicotine patch was calculated at each time point after cessation. Similar data were obtained for the 13 people who used the nicotine patch. Because there was no significant difference ($P = 0.93$) between the data for the patch and nonpatch subjects, they were combined. The mean decay of NNAL plus NNAL-Gluc per 24 h, expressed as a percentage of the baseline value, is illustrated for all 27 subjects in Fig. 5A. A similar analysis for NNAL-Gluc is shown in Fig. 5B and for NNAL in Fig. 5C. Similar results were obtained when NNAL and NNAL-Gluc were expressed per ml of urine or per mg of creatinine.

For those who did not use the nicotine patch, individual values of urinary cotinine and nicotine were determined through the 98-day time point after cessation. The mean values were expressed as a percentage of baseline, which was set at 100%. These data are summarized in Fig. 6. The 21–98-day cotinine data and the 7–98-day nicotine data are not significantly different from background levels.

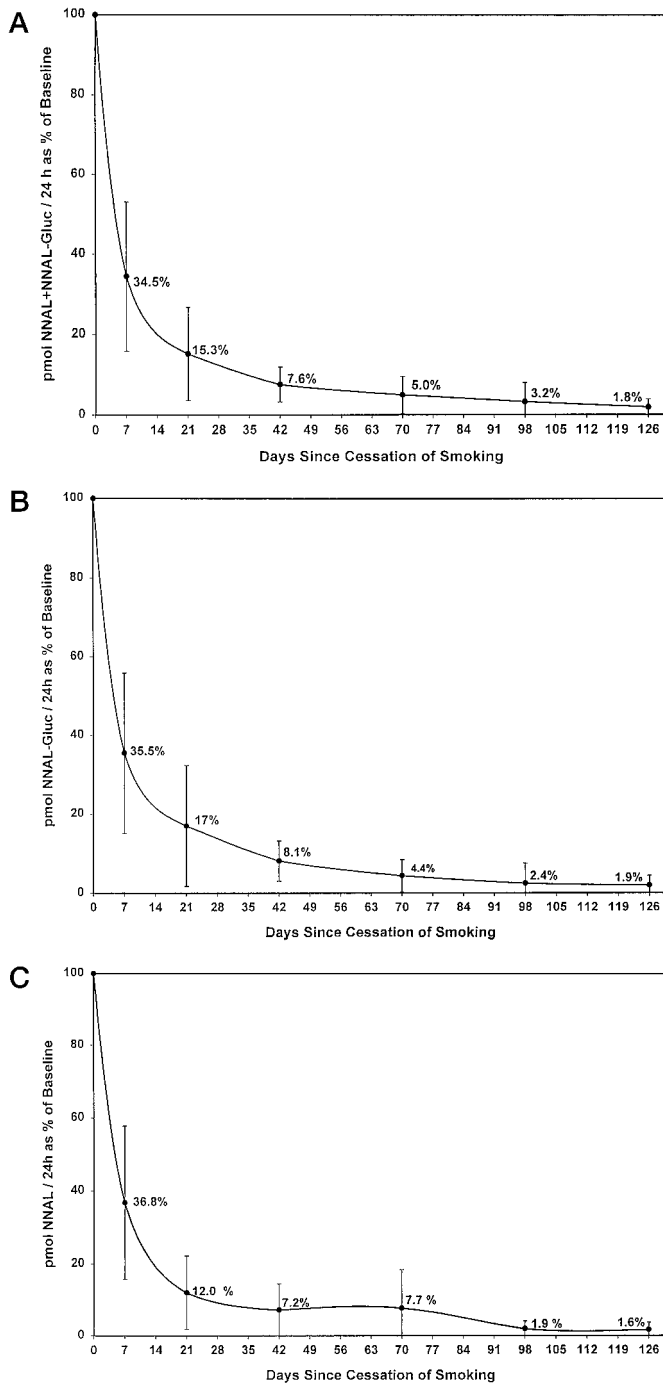


Fig. 5. Mean levels of NNAL plus NNAL-Gluc (A), NNAL-Gluc (B), and NNAL (C) in the urine of 27 subjects at various intervals after smoking cessation. Data points, percentage of mean baseline levels of NNAL plus NNAL-Gluc (pmol per 24 h), set at 100%; bars, SD.

On the basis of chemical considerations, we hypothesized that NNK could be bound as a Schiff base to blood proteins. Therefore, we isolated RBCs and plasma from four blood samples taken at baseline from active smokers. They were extracted with CH₂Cl₂, and the extracts were analyzed for NNK and NNAL. Then the remaining aqueous phase of each was treated with base or acid under conditions that would hydrolyze a Schiff base. These hydrolysates were then extracted and analyzed. NNK was not detected in any of the samples. NNAL was detected in CH₂Cl₂ extracts of unhydrolyzed plasma from three of the four smokers. Levels of NNAL were 0.052, 0.086, and

0.114 pmol/ml plasma. The corresponding levels of NNAL in the urine of these three individuals were 0.238, 0.498, and 0.352 pmol/ml, whereas those of NNAL-Gluc were 0.639, 1.39, and 0.986 pmol/ml, respectively. NNAL was not detected in CH₂Cl₂ extracts of RBCs, or in the base or acid hydrolysates of RBC or plasma.

The renal clearance of NNAL in the three smokers for whom blood and urine data were available was 9.8 ± 8.0 ml/min. Assuming that NNAL was not bound to plasma proteins, as indicated by the hydrolysis experiments, it appears that NNAL is substantially reabsorbed in the renal tubules. The total body clearance of NNAL in these subjects was 61.4 ± 35.4 ml/min, suggesting that NNAL is a relatively low-clearance compound. With the use of the average value of β found in the subjects who had quit smoking, the volume of distribution in the β -phase for these three subjects was calculated to be 3800 ± 2100 liters, indicating substantial distribution into the tissues.

The persistence of NNAL and NNAL-Gluc was also investigated in rats. Rats were treated with 2 ppm NNK in the drinking water for 28 days (chronic administration) or 2 days (acute). In the rats treated chronically, levels of NNAL and NNAL-Gluc in urine were 332 ± 30.1 pmol/ml and 371 ± 49.8 pmol/ml, respectively, at day 15, which represents 5–10% of the NNK dose, in agreement with a previous study (14). Data on the persistence of these metabolites after cessation of treatment are summarized in Table 3. In the chronically treated rats, levels of NNAL decreased rapidly, reaching <1% of the

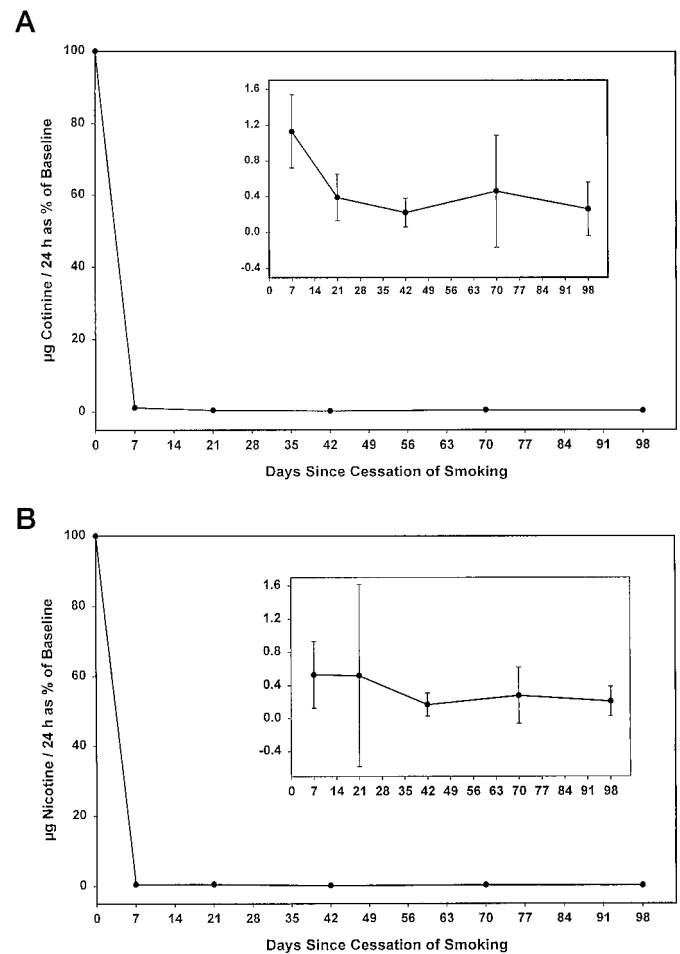


Fig. 6. Mean levels of cotinine plus cotinine-N-glucuronide (A) and nicotine plus nicotine-N-glucuronide (B) in the urine of 14 subjects at various intervals after smoking cessation. Data points, percentage of mean baseline levels; bars, SD. These subjects did not use the nicotine patch. Insets, details of data from days 7–98. Levels after day 7 are the same as those found in the urine of nonsmokers.

Table 3 Decline of NNAL and NNAL-Gluc in the urine of rats after treatment with NNK^a

Days since cessation of treatment	Chronic NNK treatment, pmol/ml (% of day 15 value)		Acute NNK treatment, pmol/ml	
	NNAL	NNAL-Gluc	NNAL	NNAL-Gluc
1	36.0 ± 11.9 (10.8)	84.9 ± 31.3 (22.9)	44.0 ± 22.0	54.1 ± 5.13
2	3.16 ± 1.58 (0.96)	38.4 ± 8.95 (10.3)	2.80 ± 0.33	17.7 ± 6.0
3	1.07 ± 0.17 (0.33)	26.6 ± 4.67 (7.2)	1.23 ± 0.58	13.9 ± 2.26
4	0.67 ± 0.32 (0.20)	28.7 ± 14.0 (7.7)	1.10 ± 0.57	9.14 ± 1.30
5	0.95 ± 0.01 (0.29)	21.9 ± 4.57 (5.9)	1.59 ± 2.75	6.80 ± 1.72

^a Groups of three male F-344 rats were given NNK (2 ppm) in the drinking water for 28 days (chronic) or 2 days (acute). NNAL and NNAL-Gluc were quantified in urine for 5 days after cessation of NNK administration (see "Materials and Methods"). In the chronically treated rats, NNAL plus NNAL-Gluc in urine were also measured on day 15; levels were 371 ± 49.8 (NNAL-Gluc) and 332 ± 30.1 (NNAL) pmol/ml.

baseline value by day 2 of cessation. However, NNAL-Gluc levels in urine declined much more slowly and were still 5.9% of baseline 5 days after cessation of treatment. The β -phase of NNAL-Gluc appeared to be prolonged in the chronically treated rats, leading to a significantly longer terminal half-life than in the acute treatment (8.06 versus 2.12 days).

DISCUSSION

The decline in urinary concentrations of NNAL and NNAL-Gluc after smoking cessation was expected, but the slow rate of decline was unexpected. The surprisingly long persistence of NNAL and NNAL-Gluc is most vividly illustrated by comparison to nicotine and cotinine. Thus, 1 week after smoking cessation, 34.5% of baseline NNAL plus NNAL-Gluc was detected in urine, whereas the corresponding values for nicotine and cotinine were 0.5 and 1.1% (Figs. 5 and 6). After 3 weeks, NNAL plus NNAL-Gluc levels in urine were still 15.3% of baseline, whereas the amounts of nicotine and cotinine were indistinguishable from background levels. The value of $t_{1/2\beta}$ for cotinine has been estimated as 16.5 h upon smoking cessation (8) whereas the corresponding values for NNAL and NNAL-Gluc are 45.2 and 39.6 days, respectively. The slow decay of NNAL and NNAL-Gluc after smoking cessation is due to their slow clearance from the body as well as the presence of a high-affinity compartment where NNK, NNAL, and/or NNAL-Gluc is/are retained or sequestered and released slowly.

The excretion rate-time profiles for NNAL and NNAL-Gluc were parallel and the fitted rate constants α and β were not significantly different for the two compounds. The elimination of NNAL-Gluc is likely to be rate controlled by its formation from NNAL. Therefore, the pharmacokinetics of NNAL effectively controls the excretion rate profile of NNAL-Gluc.

NNAL itself has a very low clearance and a large volume of distribution, the consequence of which is a terminal half-life of ~45 days. The renal clearance of NNAL is significantly lower than the glomerular filtration rate, indicating substantial reabsorption from the renal tubules. The total body clearance of NNAL is also quite low, and a comparison to hepatic liver blood flow indicates that NNAL would be considered a low-extraction ratio drug.

One of the difficulties with the pharmacokinetic analysis is knowing precisely the dose of NNAL to which the subject is exposed. The daily dose was estimated from the number of cigarettes smoked and the amount of NNK contained in cigarettes. It is well known that smoking conditions can affect NNK yield (15, 16). The conversion of NNK to NNAL is also an estimate. If the actual dose of NNAL were lower than we estimated, the clearance of NNAL would be proportionally smaller. This does not invalidate the finding of a low total body clearance for NNAL.

Although the inefficient clearance mechanisms contribute to the long half-life of NNAL, its substantial volume of distribution is probably more important. A large volume of distribution is not with-

out precedent and other basic compounds, including chloroquine (116–285 liters/kg), amiodarone (65 liters/kg), and desipramine (24–60 liters/kg) have large volumes of distribution in humans (17, 18).

NNK, NNAL, nicotine, and cotinine share some structural features. They are all 3-substituted pyridines and they all have a four-carbon chain or ring and a methyl-substituted nitrogen. All are relatively water-soluble compounds. Nicotine is a stronger base than the other three. Clearly, the pyridine ring itself is not responsible for the retention or sequestration because all four compounds have this structural feature. The carbonyl group of NNK is one structural feature that could lead to retention in the body. NNK could potentially form Schiff bases with amino groups of proteins, although there are no reports of this type of binding. We could find no evidence for reversible binding of NNK to blood proteins of smokers.

Several whole-body autoradiographic studies have been carried out after single doses of radiolabeled NNK to rats, hamsters, mice, or marmoset (19–23). In the rat, the highest labeling 4 days after injection of NNK is found in the nasal mucosa, bronchi, and trachea. Most of this radioactivity is irreversibly bound to tissue macromolecules, probably in the form of adducts that would not regenerate NNK or NNAL (1, 19). Four h and 24 h after treatment of Syrian golden hamsters with NNK, radioactivity was observed in the nasal mucosa, trachea, bronchial tree, liver, and eye melanin (21). Similar results were obtained in mice (20). In the marmoset monkey, radioactivity was concentrated in the liver, nasal mucosa, eye melanin, and ceruminous ear glands 4 h after injection of NNK (22). Binding to melanin may be a reflection of the basicity of NNK and is also observed in animals treated with nicotine, cotinine, and other basic compounds (23–25). Collectively, the presently available whole-body autoradiographic data provide little insight on the nature of the site in which NNK or NNAL is sequestered in humans. However, a recent study of NNK disposition after instillation in the canine trachea demonstrated the presence of a slow clearance phase from the trachea (26). NNK was distributed within the entire depth of the mucosa to the tracheal cartilage; a portion was conspicuously bound to the mucin component of the mucous lining layer. The slow clearance phase in this study was attributed to reversible binding to mucin (26).

The data from the acute and chronic studies in rats showed similar biphasic pharmacokinetics. However, the levels of NNAL appeared to reach background before the terminal phase was apparent. It is interesting to note that the terminal phase of NNAL-Gluc was longer in the chronic study than in the acute study. This would be consistent with a compound having a large volume of distribution. After acute dosing, relative to the blood compartment, there is not as much drug residing in the tissue compartments. Therefore, upon the cessation of the acute dosing, the elimination appears to be more rapid and less dependent upon redistribution from the tissue space. In contrast, when the tissue compartments contain more drug, as after chronic dosing, the elimi-

nation from the body will be much more dependent upon redistribution from the tissue space.

There was no evidence for endogenous formation of NNK in this study. If endogenous formation of NNK were occurring, we would have expected higher levels of NNK metabolites in nicotine patch users compared to those who did not use the patch. This was not observed. This is consistent with a previous study in rats in which we did not detect any evidence for endogenous formation of NNK after treatment of rats with nicotine and sodium nitrite (27). However, endogenous formation of *N'*-nitrosoanabasine and *N'*-nitrosoanabasine was observed in that study.

Although the retention of NNAL and NNAL-Gluc was longer than expected, the results clearly demonstrate that smoking cessation diminishes exposure to the pulmonary carcinogen NNK and that, ultimately, this substance and its metabolites are eliminated from the body. Furthermore, use of the nicotine patch does not affect the rate of elimination of NNAL and NNAL-Gluc. The decrease in carcinogen exposure upon smoking cessation will result in a decrease in risk. Because NNAL and NNAL-Gluc can be readily quantified in human urine, it is possible that measurements of these NNK metabolites could be used as a positive reinforcement device to encourage and maintain smoking cessation.

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