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Advances in Brief

Stereochemistry of Metabolites of a Tobacco-specific Lung Carcinogen in Smokers' Urine[1]

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Abstract

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific lung carcinogen, is believed to be important as a causative agent for lung cancer in smokers. NNK is extensively metabolized to its carbonyl reduction product 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which, in turn, can be glucuronidated, producing [4-methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-O-D-glucosiduronic acid (NNAL-Gluc). Metabolism of NNK to NNAL produces a chiral center. A recent study demonstrated that (*R*)-NNAL is more tumorigenic in mice than (*S*)-NNAL and that these enantiomers have substantially different metabolic pathways. Therefore, it is important to determine the stereochemistry of NNAL and NNAL-Gluc in smokers. In this study, we used chiral stationary phase-gas chromatography-nitrosamine-selective detection with confirmation by liquid chromatography-tandem mass spectrometry to determine the stereochemistry of NNAL and NNAL-Gluc in smokers' urine. The two methods agreed well. The results of analyses of urine samples from 30 smokers demonstrated that the enantiomeric distribution of NNAL in urine was 54% (*R*) and 46% (*S*) ± 7.0 (SD), whereas the diastereomeric distribution of NNAL-Gluc was 68% (*R*) and 32%(*S*) ± 8.1. These results conclusively demonstrate that both (*R*)- and (*S*)-NNAL are formed metabolically from NNK in smokers. These data are essential for furthering our understanding of the role of NNK as a cause of lung cancer in smokers.

Introduction

NNK[3] is found in substantial quantities in tobacco products and is a potent pulmonary carcinogen in rodents, primarily inducing adenocarcinoma (1–3). This tobacco-specific lung carcinogen is believed to play a role as a cause of lung cancer in smokers (1–3). NNK is rapidly metabolized in rodents and humans by carbonyl reduction, leading to NNAL (1). NNAL has a chiral center and, therefore, exists as a pair of enantiomers, (*S*)-NNAL and (*R*)-NNAL (Fig. 1). Enantiomers frequently differ in their biological effects. Recently, we demonstrated that (*R*)-NNAL is more tumorigenic than (*S*)-NNAL in A/J mouse lung; the tumorigenicity of (*R*)-NNAL was the same as that of NNK (4). Earlier studies showed that racemic NNAL is as tumorigenic as NNK in F-344 rats (5). The NNAL enantiomers are further metabolized to the corresponding diastereomeric glucuronides (*S*)-NNAL-Gluc and (*R*)-NNAL-Gluc. (*S*)-NNAL-Gluc is not tumorigenic in mice (4). The NNAL enantiomers, like NNK, undergo metabolic activation by α-hydroxylation, leading to DNA adduct formation, a critical step in cancer induction by these compounds (Fig. 1). In several studies, we have quantified NNAL and NNAL-Gluc in human urine (1, 6–9). These metabolites are excellent biomarkers for uptake of NNK in smokers, snuff-dippers, and people exposed to environmental tobacco smoke (1). However, no information was available on the enantiomeric composition of NNAL or the diastereomeric composition of NNAL-Gluc in smokers' urine. This is important in view of the higher tumorigenicity of (*R*)-NNAL than (*S*)-NNAL. Therefore, in this study, we used CSP-GC-TEA, as well as LC-MS/MS, to address this question.

Materials and Methods

Urine samples were obtained from 30 smokers (12 males) who were participating in various ongoing studies of NNK metabolism in humans. All studies were approved by the University of Minnesota Research Subjects' Protection Program Institutional Review Board: Human Subjects Committee.

(*S*)- and (*R*)-NNAL were prepared by hydrolysis of the corresponding α-methoxy-α-(trifluoromethyl)phenyl acetates (10). (*R*)-MBIC was obtained from Aldrich Chemical Co. (Milwaukee, WI). (*R*)-NNAL-(*R*)-MBIC and (*S*)-NNAL-(*R*)-MBIC were prepared as described (11). [CD_3]NNAL was prepared by $NaBH_4$ reduction of [CD_3]NNK (12).

The GC-TEA method for analysis was carried out on 10 ml of urine essentially as described previously (6), except that a 30 m × 0.25-mm inside diameter, 0.25-μm film thickness Cyclosil-B CSP-GC column (J & W Scientific, Folsom, CA) was used. This column has 30% heptakis-(2,3-di-*O*-methyl-6-*O*-*t*-butyldimethylsilyl)-β-cyclodextrin chiral selector physically dispersed in the DB-1701 stationary phase [(14% cyanopropylphenyl)methylpolysiloxane]. The column was operated with a 2 m × 0.53-mm inside diameter-deactivated fused silica precolumn. The initial temperature of the oven was 60°C for 2 min, then it was programmed at 12°C/min until 166°C, then held for 85 min. The GC was operated in the pulse splitless mode, and the injection port temperature was 225°C. The carrier gas was helium at 1 ml/min.

For LC-MS/MS analysis, the sample preparation steps were identical to those described for our GC-TEA method, up until the point of silylation (6). One exception was the use of [CD_3]NNAL (4 ng) as internal standard instead of *iso*-NNAL. The purified sample from each of the NNAL and NNAL-Gluc fractions was allowed to react with (*R*)-(+)-MBIC (80 μl) in 2 ml of benzene containing 120 μl of triethylamine at 70°C overnight. The benzene was removed in a stream of N_2, then 2 ml of CH_3CN/H_2O:7/3 was added. After standing overnight, the supernatant was removed, transferred to a 4-ml vial, and rinsed with CH_3CN. The mixture was concentrated to dryness *in vacuo* and redissolved in 100 μl of CH_3CN/H_2O:7/3 for HPLC purification on a 4.6 × 250-mm Microsorb-MV C18 column (Varian Assoc., Walnut Grove, CA) with elution by A [10 mM NH_4OAc (pH 7.0)] and B (CH_3CN). The program was as follows: initial conditions 66% A, 34% B for 20 min, then a linear gradient in 5 min to 5% A, 95% B at 1 ml/min. Eluent at the retention time of standards was collected, concentrated to dryness, and redissolved in 15 μl of CH_3CN/H_2O:7/3 for LC-MS/MS analysis on a Finnigan TSQ 7000 instrument operated in the positive ion atmospheric pressure chemical ionization-MS/MS mode, with monitoring of the transition *m/z* 357 (M+1)→162 for the analyte and *m/z* 360→165 for the internal standard. HPLC separation was achieved with two Microsorb-MV C18 columns with isocratic elution using

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[3] The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; CSP-GC-TEA, chiral stationary phase-gas chromatography-nitrosamine-selective detection; LC-MS/MS, liquid chromatography-tandem mass spectrometry; (*R*)-MBIC, (*R*)-(+)-α-methylbenzyl isocyanate; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; [CD_3]NNAL, 4-(trideuteromethylnitrosamino)-1-(3-pyridyl)-1-butanol; NNAL-Gluc, [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-O-D-glucosiduronic acid; *iso*-NNAL, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; (*R*)-NNAL-(*R*)-MBIC, 4-(methylnitrosamino)-1(*R*)-[(*R*)-α-methylbenzylcarbamoyl]-1-(3-pyridyl)butane; HPLC, high-performance liquid chromatography.

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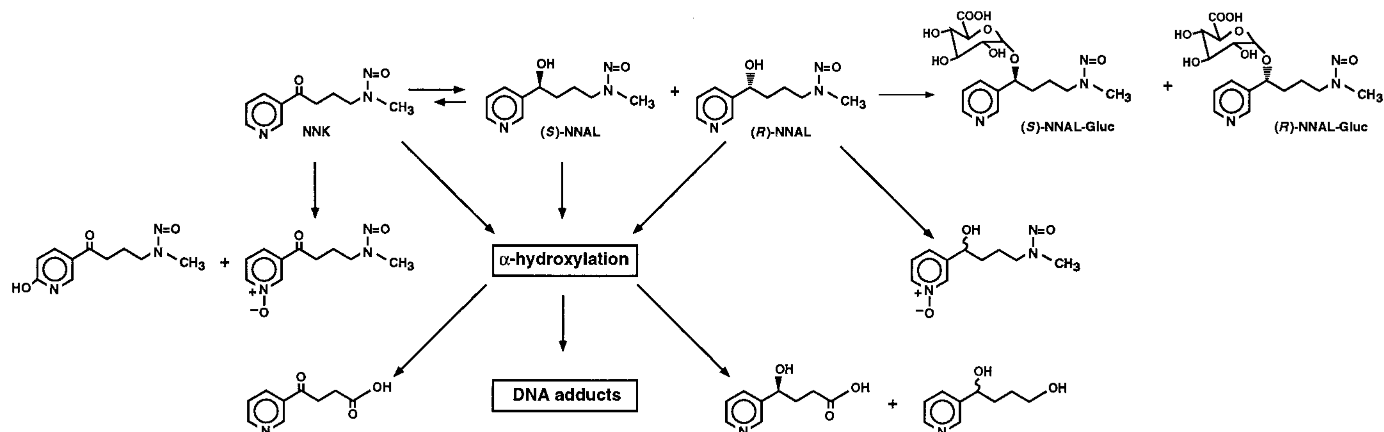

Fig. 1. Overview of NNK metabolism to NNAL enantiomers, NNAL-Gluc diastereomers, and other urinary metabolites. For further detail, see Ref. 1.

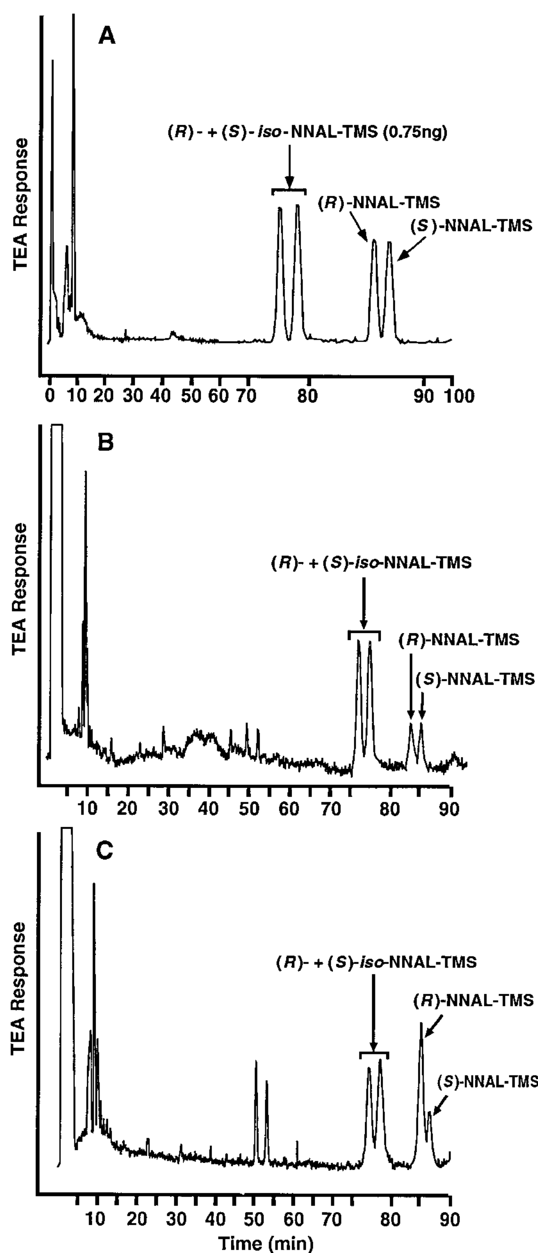

Fig. 2. CSP-GC-TEA chromatograms of: *A*, standard racemic *iso*-NNAL (internal standard), (*R*)-NNAL, and (*S*)-NNAL as their trimethylsilyl (*TMS*) ether derivatives; *B*, NNAL fraction of a smoker's urine; *C*, NNAL-Gluc fraction of a smoker's urine.

65% 10 mM NH_4OAc and 35% CH_3CN at 1 ml/min. MS conditions were the same as described previously (13).

Results

In the GC-TEA method for analysis of NNAL and NNAL-Gluc in human urine, two fractions are produced: one containing free NNAL and the other containing NNAL that has been released from NNAL-Gluc by hydrolysis with β-glucuronidase (6). We have previously shown that hydrolysis of both (*S*)-NNAL-Gluc and (*R*)-NNAL-Gluc is complete under our conditions (6). Free or released NNAL is then converted to its trimethylsilyl ether (NNAL-TMS), which is analyzed by GC-TEA. Using CSP-GC-TEA, baseline resolution of (*S*)- and (*R*)-NNAL-TMS standards, as well as (*S*)- and (*R*)-*iso*-NNAL internal standard, is achieved (Fig. 2*A*). A typical chromatogram of the NNAL fraction from a smoker's urine is illustrated in Fig. 2*B*, whereas Fig. 2*C* shows a representative chromatogram of NNAL released from the NNAL-Gluc fraction.

We used LC-MS/MS to confirm the CSP-GC-TEA results. For this

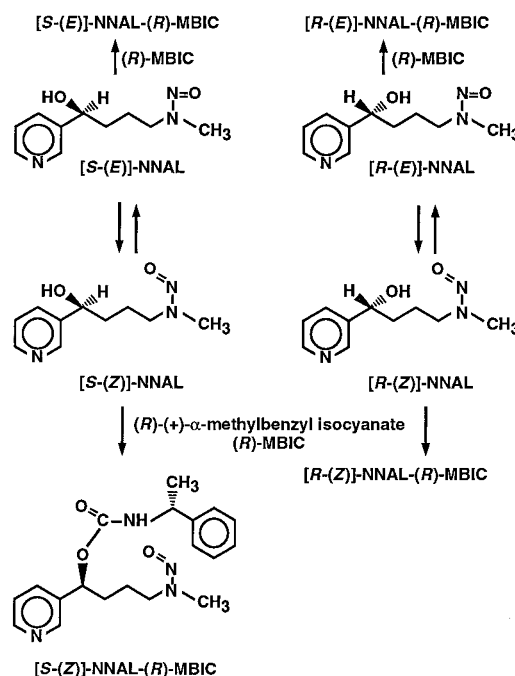

Fig. 3. Structures of enantiomers and rotamers of NNAL and their diastereomeric derivatives produced by reaction with (*R*)-MBIC.

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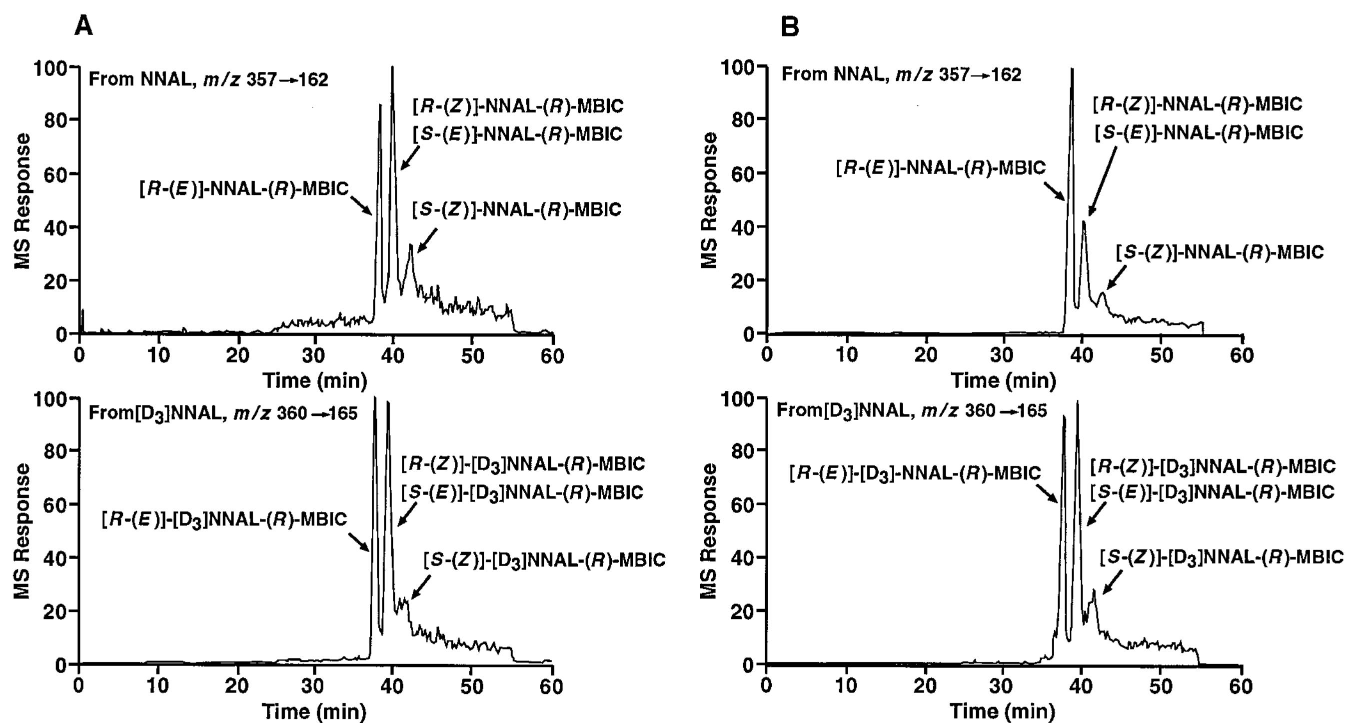

Fig. 4. LC-MS/MS chromatogram of NNAL fraction (*A*) and NNAL-Gluc fraction (*B*) of a smoker's urine. *Top*, selected reaction monitoring for the analyte at *m/z* 357 → 162. *Bottom*, corresponding analysis of the internal standard.

analysis, we again separated the urine into fractions containing free NNAL and NNAL-Gluc. The latter was hydrolyzed with β-glucuronidase. Released NNAL was derivatized with (*R*)-MBIC, producing the diastereomeric carbamates (*S*)-NNAL-(*R*)-MBIC and (*R*)-NNAL-(*R*)-MBIC. Because of restricted rotation around the N-N=O bond, (*E*)- and (*Z*)- rotamers of each diastereomer are observed, as illustrated in Fig. 3. Thus, four products are produced in this reaction, and all can potentially be separated by HPLC. The rotamers are not observed in the GC analysis due to the high temperature used. In practice, we observed only three peaks when standards were analyzed under our conditions because [*R*-(*Z*)]-NNAL-(*R*)-MBIC and [*S*-(*E*)]-NNAL-(*R*)-MBIC coeluted. This does not present a problem in quantitation because the contribution of the [*R*-(*Z*)]-NNAL-(*R*)-MBIC isomer can readily be calculated and subtracted. Representative chromatograms from the NNAL fraction and the NNAL-Gluc fraction of a smoker's urine are illustrated in Fig. 4, *A* and *B*. The chromatograms are consistent with expectations based on the GC-TEA results. The peaks corresponding to [*R*-(*E*)]-NNAL-(*R*)-MBIC and [*S*-(*E*)]-NNAL-(*R*)-MBIC were about the same size in the free NNAL fraction (Fig. 4*A*), whereas the former was about twice as great as the latter in the NNAL-Gluc fraction (Fig. 4*B*). Eight samples were analyzed by both methods. For the percentage of (*R*)-NNAL, the results were as follows: 52% ± 10 (SD) by CSP-GC-TEA and 51% ± 7.9 by LC-MS/MS. For the percentage of (*R*)-NNAL-Gluc, the results were as follows: 69% ± 2.7 by CSP-GC-TEA and 64% ± 5.3 by LC-MS/MS. These results demonstrate that the two methods agree well.

We analyzed 30 urine samples from smokers by CSP-GC-TEA because this method is operationally simpler than LC-MS/MS. Mean levels of total NNAL and NNAL-Gluc were 0.752 ± 0.441 (SD) pmol/ml and 1.80 ± 1.37 pmol/ml, respectively, similar to previously reported levels (6, 9). Frequency distributions of the enantiomeric composition of NNAL and diastereomeric composition of NNAL-Gluc are illustrated in Fig. 5, *A* and *B*. Two of the NNAL samples could not be quantified due to poor resolution. Similar to the initial sample of eight described above, the percentage of (*R*)-NNAL was 54 ± 7.0 ($n = 28$) and the percentage of (*R*)-NNAL-Gluc was 68 ± 8.1 ($n = 30$). The distribution of values seems to be normal. There was no effect of gender, number of cigarettes smoked, or absolute level of NNAL and NNAL-Gluc on the enantiomeric or diastereomeric composition.

Discussion

These results conclusively demonstrate that both (*R*)-NNAL and (*S*)-NNAL are formed metabolically from NNK in smokers. This is critical information for assessing the contribution of NNK to cancer risk in smokers because studies in mice show that the tumorigenic activities of these two enantiomers are different, with (*R*)-NNAL being significantly more tumorigenic than (*S*)-NNAL (4). It is not clear at present whether similar relative tumorigenic activities of the NNAL enantiomers would be observed in humans.

The glucuronidation of racemic NNAL is different in rodents and primates. In rat liver microsomes, glucuronidation of racemic NNAL produces mainly (*S*)-NNAL-Gluc (14). In A/J mice treated with racemic NNAL, (*S*)-NNAL-Gluc is the major diastereomer observed in urine (4). In patas monkey liver microsomes, racemic NNAL is converted mainly to (*R*)-NNAL-Gluc (4). Moreover, (*R*)-NNAL-Gluc is the major diastereomer observed in the urine of patas monkeys treated with NNK (10, 11). On the basis of these observations, we would expect humans to glucuronidate (*R*)-NNAL more extensively than (*S*)-NNAL. Our data seem to be consistent with this hypothesis and suggest that more (*R*)-NNAL than (*S*)-NNAL is produced from NNK in smokers. However, additional studies are required to confirm this because the relative extents of α-hydroxylation or pyridine-*N*-oxidation of (*R*)- and (*S*)-NNAL in humans are presently unknown.

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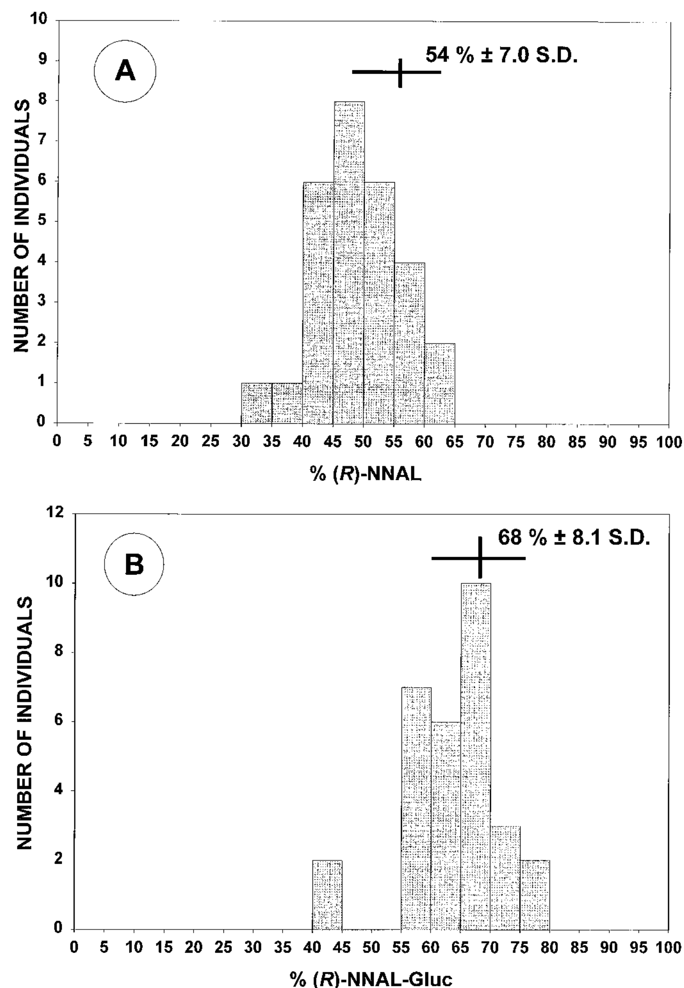

Fig. 5. Frequency distribution of (*R*)-NNAL (*A*) and (*R*)-NNAL-Gluc (*B*) in smokers' urine.

One previous study examined the levels of (*R*)- and (*S*)-NNAL-Gluc in the urine of Sudanese people who used toombak, a smokeless tobacco product with exceptionally high levels of NNK (15). The uptake of NNK in these individuals was about 70 times as great as in smokers. The NNAL-Gluc diastereomers were analyzed by collection of the appropriate regions from HPLC, treatment with β-glucuronidase, and GC-TEA analysis. The ratio (*R*)-NNAL-Gluc:(*S*)-NNAL-Gluc was 1.9 ± 0.5 ($n = 7$), similar to the data obtained here. However, because of the unusually high NNK dose and the different route of administration, it was not clear whether these results would be generalizable to smokers.

In summary, this study clearly demonstrates that both (*R*)- and (*S*)-NNAL are metabolites of NNK in smokers. The amounts of (*R*)- and (*S*)-NNAL in urine are about the same, whereas there is twice as much (*R*)-NNAL-Gluc as (*S*)-NNAL-Gluc. These results add a new dimension to our understanding of human metabolism of the tobacco-specific lung carcinogen NNK.

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