METABOLISM AND DISPOSITION OF 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE (NNK) IN RHESUS MONKEYS

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ABSTRACT:

Metabolism and disposition of the tobacco-specific N-nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a potent rodent lung carcinogen, were studied in rhesus monkeys. In three males receiving a single i.v. dose of [5-3H]NNK (0.72 mcCi; 4.6–9.8 μg/kg), urine was collected for 10 days. Within the first 24 h, 86.0 ± 0.7% of the dose was excreted. NNK-derived radioactivity was still detectable in urine 10 days after dosing (total excretion, 92.7 ± 0.7%). Decay of urinary radioactivity was biexponential with half-lives of 1.7 and 42 h. Metabolite patterns in urine from the first 6 h closely resembled those reported previously for patas monkeys; end products of metabolic NNK activation represented more than 50% of total radioactivity. At later time points, the pattern shifted in favor of NNK detoxification products (60–70% of total radioactivity in urine), mainly 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its O-glucuronide conjugates. One female rhesus monkey received a single i.v. dose of [5-3H]NNK (1.72 mcCi; 28.4 μg/kg) under isoflurane anesthesia; biliary excretion over 6 h (0.6% of the dose) was 10 times less than predicted by our previously reported rat model. No preferential excretion of NNK glucuronide was observed in monkey bile. Collectively, these results suggest that the rhesus monkey could be a useful model for NNK metabolism and disposition in humans.

In his comprehensive review, Hecht (1998) compiled a large amount of data available on the biochemistry, carcinogenicity, and other biological properties of tobacco-specific N-nitrosamines, particularly N′-nitrosornicotine (NNN)1 and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). However, major areas still require further research. NNK is a strong lung carcinogen that induces adenocarcinomas in rats, mice, and hamsters, independent of the route of administration. The lifetime NNK dose in a smoker is claimed to be similar to the lowest total NNK dose that induces lung tumors in rats. Thus, Hecht (1998) concluded that NNK may play an important role in the etiology of adenocarcinoma. Such quantitative risk estimations may not be valid if major differences occur between rodents and humans in NNK metabolism and disposition.

Five major metabolic transformations of NNK have been observed (Fig. 1): carbonyl reduction to give 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanal (NNAL), glucuronidation of NNAL, and, for both NNK and NNAL, pyridine N-oxidation and α-hydroxylation (hydroxylation of carbons adjacent to the N-nitroso group). Enzymology of these reactions has not been fully characterized. Pyridine N-oxidation and α-hydroxylation are mediated mainly by cytochrome P-450 isozymes (Crespi et al., 1991; Smith et al., 1995; Patten et al., 1996; Smith et al., 1997); 11-β-Hydroxysteroid dehydrogenase may be involved in carbonyl reduction of NNK (Maser et al., 1996). α-Hydroxylation is the major route of metabolic activation of NNK and NNAL, whereas pyridine N-oxidation of NNK and NNAL and glucuronidation of NNAL serve as detoxification pathways. For risk estimation it is necessary to determine the balance of these metabolic pathways in humans compared with rodents. Unfortunately, urinary metabolites representing end products of NNK and NNAL α-hydroxylation, hydroxy and keto acids, are not specific for these two carcinogens but are also metabolites of NNN and, even more important, of nicotine (Hecht, 1998).

In a first attempt, protein and DNA adducts retaining the pyridyl-oxobutyl moieties were investigated as candidates for biomonitoring uptake and metabolic activation of NNK and NNN (Carmella and Hecht, 1987). However, the hemoglobin adduct resulting from α-methyl hydroxylation of NNK and releasing keto alcohol upon alkaline hydrolysis cannot serve as a dose-dependent biomarker (Carmella et al., 1990; Falter et al., 1994; Branner et al., 1998). Corresponding DNA adducts were detected in 1- to 2-mg samples of human lung in a pilot study (Foiles et al., 1991) but not in another study using only 0.3 mg of DNA (Blömeke et al., 1996).

In another approach, NNK metabolites retaining the N-nitroso moiety, NNAL and its stereoisomeric O-glucuronides, were detected in urine of smokers and nonsmokers and correlated closely with...
cotinine, a well established biomarker of tobacco smoke exposure (Hecht et al., 1993a; Meger et al., 1996; Parsons et al., 1998). Based on knowledge of metabolic studies in rodents, this was unexpected. In rats and mice, urinary excretion of NNAL and \([4\text{-}\text{(methylnitr}
\text{osamino)}\text{-1\text{-}(3\text{-pyridy})\text{-but-1-yl}}]\text{-}\text{O}\text{-D-glucosiduronic\ acid (NNAL-Gluc) was quantitatively unimportant at low doses of NNK (Morse et al., 1990). However, patas monkeys, even at a 10-fold lower dose, excreted 20\% of the dose as NNAL glucuronides (Hecht et al., 1993b). In contrast to rodents, two stereoisomers, NNAL-Gluc(I\text{+II}), rather than one, were detected in monkey and human urine. In smokers’ urine, the sum of NNAL and NNAL-Gluc(I\text{+II}) represented 40 to 100\% of the estimated NNK uptake, 1.6 to 4.7 \(\text{mg, with cigarette mainstream smoke (Carmella et al., 1993). Two explanations for this discrepancy between rodents and primates are conceivable. 1) A species-dependent difference exists in NNK metabolism, detoxification pathways being favored in primates compared with rodents (Hecht et al., 1993b). This species difference may become even more pronounced at lower concentrations that approach doses expected in smokers. 2) In rats, significant biliary excretion of NNAL-Gluc occurs at a low dose, 0.145 \(\text{mg of NNK/kg (Schulze et al., 1992), at which urinary excretion was negligible (Morse et al., 1990). It was suggested that in rodents, NNAL-Gluc undergoes enterohepatic recirculation because it does not appear in equivalent amounts in urine and feces. According to Hirom et al. (1977), for organic anions such as glucuronides, the minimum molecular weight at which biliary elimination becomes appreciable is about 325 \pm 50 for rats and about 50 for humans. Therefore, NNAL-Gluc with a molecular weight of 385 may be eliminated in primates mainly through the kidney, rather than in bile.}

In the present experiments with rhesus monkeys the profile of urinary metabolites was observed for 10 days after a single i.v. dose of NNK. In addition, biliary excretion of NNK and its metabolites was measured in one monkey.

**Materials and Methods**

**Chemicals.** [5\text{-}\text{H}]NNK (2.4 C\text{Ci/mmol; radiochemical purity \geq 99\%}) was obtained from Chemsyn Science Laboratories (Lenexa, KS). Unlabeled NNK metabolite standards were kindly donated by Dhimant H. Desai and Shantu Amin (American Health Foundation, Valhalla, NY). \(\text{\beta-Glucuronidase (type IX) and saccharic acid 1,4-lactone were purchased from Sigma Chemie GmbH (Deisenhofen, Germany). All other chemicals that were either HPLC or analytical grade were obtained from Merck (Darmstadt, Germany).}

**Apparatus.** Radioactive samples were counted on a Beckman LS-3133T (Beckman Instruments Inc., Fullerton, CA) or a Packard 2500 TR (Packard, Frankfurt, Germany) liquid scintillation counter using DuPont Formula-989 (NEN Research Products, Boston, MA) or Ultima Gold XR (Packard) scintillation mixture. HPLC was carried out with a Gilson 231 autosampler (Abimed, Düsseldorff, Germany) and a Gynkotek 480 high-precision pump (Gynkotek, Germering, Munich) equipped with a UV detector (Gynkotek model UVD 160) operated at 234 and 254 nm and a Ramona 93 on-line radioactivity monitor (Raytest, Straubenhardt, Germany) operated either with a solid-phase scintillator (CaF, 0.25-ml cell volume) or by liquid scintillation counting after mixing with 4 volumes of Monofluor (National Diagnostics, Manville, NJ) scintillation mixture.

**Animal Experiments.** Four colony-reared rhesus monkeys (Macaca mulatto) were used. Three were 8-year-old males weighing 6.4, 7.5, and 13.6 kg, respectively. The fourth was a 19-year-old female weighing 5.2 kg. They were maintained individually in American Association for the Accreditation of Laboratory Animal Care-accredited housing in the primate facility of the Department of Comparative Medicine at the Pennsylvania State University College of Medicine. The temperature was maintained at 22 \(\pm 1\) °C; the relative humidity was between 40 and 60\% and fresh air was exchanged 100\% from 18 to 22 times every hour. The photoperiod was controlled to provide light from 8:00 AM to 8:00 PM. The rhesus monkeys received water ad libitum.
and Purina Monkey Chow no. 5038. They had not been exposed previously to nicotine or other chemical compounds or been involved in any procedures that might influence NNK metabolism. All animal experiments were approved by The Pennsylvania State University Institutional Animal Care and Use Committee.

Under light ketamine anesthesia, the three males were infused i.v. through the saphenous vein with a single dose of 0.72 mCi of [5-3H]NNK in 0.9 ml of sterile saline corresponding to 9.8, 8.3, and 4.6 μg/kg, respectively. The animals were placed in stainless steel metabolism cages, and urine was collected on dry ice 6 and 24 h after injection and then on 9 subsequent days, after which they were returned back to the colony. After thawing, the urine samples were weighed and aliquots were taken for determination of total radioactivity. The remaining samples were stored at −80°C and shipped to Munich on dry ice for analysis of NNK metabolites.

The female rhesus monkey was lightly anesthetized by injection of ketamine, 100 mg i.m., and prepared for aseptic surgical procedures. The anesthesia was maintained with isoflurane through an endotracheal tube. The animal was supplied with a heparinized saline catheter in the left carotic artery to collect blood periodically. The gall bladder was ligated with 4-0 silk and the common bile duct was cannulated with a 14-gauge polyethylene tube for continuous collection of bile into preweighed Eppendorf tubes, which were changed every half hour. Unfortunately, a catheter placed in the urethra slipped out and, therefore, urine was not collected quantitatively. [5-3H]NNK, 1.72 mCi (28.4 μg/kg) in 1.7 ml saline, was infused through the left saphenous vein. Serial blood samples in heparinized tubes were centrifuged immediately after collection, plasma was taken off, and the remaining blood cells were washed twice with equal volumes of saline. After 6 h the animal was sacrificed using pentobarbital i.v. (100 mg/kg). Immediately after death, 25 ml of gastric juice was collected from the stomach. All samples were stored at −80°C and shipped to Munich on dry ice for analysis of total radioactivity and NNK metabolites.

**Analysis of Metabolites.** Urine samples from the first 24 h after NNK treatment were centrifuged in Eppendorf microvials at 14,000 rpm and up to 950 μl of the supernatant was analyzed by HPLC. All other liquid samples were concentrated and purified by methanol precipitation as described by Staretz and Hecht (1995) with modifications. A 5- to 10-fold excess of methanol was added to the samples, which were kept overnight at 4°C. After centrifugation at 14,000 rpm, the supernatant was evaporated in a vacuum concentrator (Bachofer, Reutlingen, Germany). The pellet was washed once more with methanol and centrifuged, and the supernatant was given on the residue of the first methanol extraction. After concentration to dryness by vacuum centrifugation the residue was dissolved in 20 mmol of sodium/potassium phosphate buffer, pH 6.0, for HPLC analysis.

All samples were analyzed by reversed-phase HPLC using a 4×250-mm LiChrosphere 60 RP-select B 5-μm column fitted with a 4×4-mm guard column (Merck, Darmstadt, Germany) with a 20 mM sodium/potassium phosphate (pH 6)/acetonitrile gradient increasing after an initial hold of 3 min from 0 to 30% acetonitrile in 37 min, to 50% acetonitrile in 2 min, and back to 0% acetonitrile in 2 min.

Radioactive metabolites were confirmed by cochromatography, with unlabeled reference compounds detected by UV. The presence of NNAL glucuronide in urine was confirmed by treatment of samples with β-glucuronidase at 37°C overnight with or without saccharic acid 1,4-lactone (Schulze et al., 1992).

**Data Analysis.** Pharmacokinetics of NNK and its metabolites were analyzed by conventional methods using GraphPad Prism version 2.01 (GraphPad Software Inc., San Diego). Concentration versus time data were used for estimation of plasma pharmacokinetics. For urinary excretion of NNK and its metabolites, sigma minus plots were taken to analyze NNK disposition (Kyeremaen et al., 1982). Differences between the means for NNK metabolites depending on the time after dosing were tested for by the Student’s t test for unpaired data using WinSTAT (G. Greulich Software, Staufen, Germany).

### Results

**Renal Elimination of NNK and Its Metabolites.** In three male rhesus monkeys treated with 5 to 10 μg/kg [5-3H]NNK by i.v. injection, urine was the major route of excretion, releasing 74 to 80% of the total radioactivity administered within the first 6 h and increasing to 85 to 87% during the first day. As shown in Fig. 2, urinary excretion of the radiolabel continued during the following 9 days at a low but measurable level. Ten days after i. v. administration, renal clearance accounted for 91 to 94% of the dose (Table 1). Two characteristic chromatograms obtained from urine fractions collected in the first 6 h and on the ninth day after injection are illustrated in Fig. 3. The main radioactive peaks in both runs are hydroxy acid, keto acid, NNAL-Gluc(I+II), and, on the ninth day, NNAL. The N-oxides of NNK and NNAL as well as the primary α-hydroxylation products keto alcohol and diol were minor metabolites throughout the experiment. The nature of NNAL-Gluc(I+II) was confirmed by incubation of urine samples with glucuronidase (Schulze et al., 1992), resulting in the disappearance of the peaks and a corresponding increase of the NNAL peak in the chromatograms. The effect of the glucuronidase could be blocked by coincubation of urine samples with the specific inhibitor saccharic acid 1,4-lactone.

A time-dependent shift occurred in the distribution of NNK metabolites in urine. In Fig. 4 the sum of metabolites stemming from α-hydroxylation of NNK and NNAL, hydroxy acid, keto acid, diol, and keto alcohol (Fig. 1) is plotted against the sum of metabolites that have not undergone metabolic activation because they have retained the N-nitroso group, NNAL-N-oxide, NNK-N-oxide, and NNAL and its glucuronides. The percentage of α-hydroxylation products is significantly higher than the sum of products retaining the N-nitroso group only in urine collected during the first 6 h after NNK administration, when the bulk of radioactivity has been eliminated (76.7 ± 1.8% of dose). Thereafter, the relationship is reversed and the difference again becomes significant on the fourth day. Whereas the sum of N-oxides remains at about 10% of the total radioactivity excreted per day, the percentage of NNAL and its glucuronides increases from 35% to 50 to 60%.

Sigma minus plots of urinary metabolite excretion revealed a two-phase exponential decay in male rhesus monkeys with half-lives of 1.4 to 1.8 h and 39 to 50 h for the major NNK metabolites (Fig. 5; Table 1). These half-lives agree with the decay of total radioactivity in urine.
(half-lives of 1.7 and 42 h with the σ minus method). For the minor metabolites, similar half-lives were obtained with the N-oxidation products. The deviation of half-lives for the primary α-hydroxylation products, diol and keto alcohol, should be interpreted with caution because of the difficulty in measuring these minor metabolites accurately in urine fractions collected at later times after NNK administration.

Kinetics of NNK and Its Metabolites in Bile and Plasma. In one female rhesus monkey that received i.v. under isoflurane anesthesia 28.4 μg/kg [5-3H]NNK, bile was only a minor route of excretion, containing 0.62% of the total radioactivity administered within 6 h. This result apparently conflicts with recent rat data from Schulze et al. (1992), showing 10-fold-higher biliary excretion (Fig. 6). Four to 6 h after NNK administration, a much more complex metabolite pattern was observed in the bile of the monkey compared with that of rats (Fig. 7). Throughout the 6-h sampling period, products of NNK α-hydroxylation contributed about one-third of total metabolites in bile, whereas NNAL and its glucuronides increased from a low of 23% after 30 min to a high of 60% after 2 h. Unchanged NNK was

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TABLE 1

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>% Recovery of Dose After 24 h</th>
<th>% Recovery of Dose After 10 days</th>
<th>T_{1/2} (h)</th>
<th>α</th>
<th>β</th>
<th>r^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxy acid</td>
<td>28.7 ± 1.6^d</td>
<td>30.5 ± 1.9</td>
<td>1.8</td>
<td>39</td>
<td>0.989</td>
<td></td>
</tr>
<tr>
<td>Keto acid</td>
<td>13.7 ± 2.0</td>
<td>14.3 ± 2.0</td>
<td>1.4</td>
<td>43</td>
<td>0.958</td>
<td></td>
</tr>
<tr>
<td>NNAL-Gluc(I)</td>
<td>0.8 ± 0.6</td>
<td>1.1 ± 0.8</td>
<td>1.6</td>
<td>50</td>
<td>0.788</td>
<td></td>
</tr>
<tr>
<td>NNAL-Gluc(II)</td>
<td>19.2 ± 6.3</td>
<td>19.8 ± 6.5</td>
<td>1.6</td>
<td>35</td>
<td>0.801</td>
<td></td>
</tr>
<tr>
<td>NNAL-N-oxide</td>
<td>3.9 ± 1.2</td>
<td>4.3 ± 1.3</td>
<td>0.2</td>
<td>49</td>
<td>0.663</td>
<td></td>
</tr>
<tr>
<td>Diol</td>
<td>1.3 ± 0.7</td>
<td>1.4 ± 0.6</td>
<td>0.2</td>
<td>49</td>
<td>0.663</td>
<td></td>
</tr>
<tr>
<td>Keto alcohol</td>
<td>2.2 ± 0.3</td>
<td>2.3 ± 0.3</td>
<td>0.2</td>
<td>49</td>
<td>0.663</td>
<td></td>
</tr>
<tr>
<td>NNAL</td>
<td>9.4 ± 3.2</td>
<td>11.4 ± 3.1</td>
<td>1.8</td>
<td>48</td>
<td>0.842</td>
<td></td>
</tr>
<tr>
<td>NNK</td>
<td>1.2 ± 0.9</td>
<td>1.3 ± 0.9</td>
<td>1.5</td>
<td>49</td>
<td>0.441</td>
<td></td>
</tr>
<tr>
<td>Σ of α-hydroxylation^b</td>
<td>45.9 ± 2.1</td>
<td>48.5 ± 2.1</td>
<td>1.7</td>
<td>42</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td>Σ of N-oxidation^c</td>
<td>8.1 ± 2.1</td>
<td>8.9 ± 2.2</td>
<td>1.7</td>
<td>42</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td>NNAL + NNAL-Gluc</td>
<td>29.4 ± 3.9</td>
<td>32.2 ± 4.4</td>
<td>1.7</td>
<td>42</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>86.0 ± 0.7</td>
<td>92.7 ± 0.7</td>
<td>1.7</td>
<td>42</td>
<td>0.999</td>
<td></td>
</tr>
</tbody>
</table>

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Footnotes:

^a Three male rhesus monkeys (6.4, 7.5, and 13.6 kg) received i.v. injections of 0.72 mCi of [5-3H]NNK (9.8, 8.3, and 4.6 μg/kg). Urine was collected for 10 days and analyzed by HPLC as described in Materials and Methods.

^b Including hydroxy acid, keto acid, diol, and keto alcohol.

^c Including NNAL-N-oxide and NNK-N-oxide.

^d Percentage of dose, mean ± S.E.

^e Kinetic analysis by the sigma minus method using a two-phase exponential decay model.

^f Sum of NNAL-Gluc(I) and NNAL-Gluc(II) taken for analysis.

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Fig. 3. Comparison of the urinary profile of [5-3H]NNK metabolism in rhesus monkeys 6 h and 9 days after i.v. injection.

Fig. 4. Distribution of metabolite groups in urine after i.v. injection of [5-3H]NNK.

Metabolites resulting from α-hydroxylation, hydroxy acid, keto acid, diol, and keto alcohol are plotted against metabolites retaining the N-nitroso function, NNAL, NNAL-Gluc(I+II), NNAL-N-oxide, and NNK-N-oxide. Values are the mean ± S.E. of three animals. *p < .05; **p < .01; ***p < .001.
detectable in bile only during the first hour after [5-^3H]NNK administration (Table 2).

Excretion of total radioactivity in bile reached a plateau at 30 to 60 min and demonstrated a one-phase exponential decay \( (T_{1/2} = 23 \text{ min}, r^2 = 0.9911) \) during the succeeding 5 h (Fig. 8). In contrast, disposition of NNK-derived radioactivity in plasma exhibited a two-phase exponential decay with \( T_{1/2a} = 17 \text{ min} \) and \( T_{1/2B} = 3.8 \text{ h} \) \( (r^2 = 0.9938) \).

NNK metabolites could be determined in plasma up to 3 h after

![Fig. 5. Sigma minus plots of urinary excretion of NNK and nine metabolites in three male rhesus monkeys after i.v. injection of [5-^3H]NNK; mean ± S.E.](image)

![Fig. 6. Cumulative biliary excretion of NNK in a female rhesus monkey after i.v. injection of 28.4 μg/kg [5-^3H]NNK and in six rats after i.p. injection of 144 μg/kg [1-^14 C]NNK.](image)

Rat data were taken from Schulze et al. (1992) with permission.

![Fig. 7. Comparison of the biliary profile of [5-^3H]NNK metabolism in a rhesus monkey after 6 h and of [1-^14 C]NNK metabolism in rats after 4 h.](image)

Rat data were taken from Schulze et al. (1992) with permission.
NNK and Its Metabolites in Gastric Juice. Six hours after treatment of a female rhesus monkey with 28.4 μg/kg [5-3H]NNK by i.v. injection, a sample of gastric juice was taken and analyzed with HPLC (Fig. 9). NNAL is the predominant metabolite. Because of the low concentrations, the contribution of further metabolites and of NNK itself to total radioactivity in gastric juice could not be determined accurately.

Discussion

The field of human biomonitoring developed to estimate individual health risks allows assessment not only of exposure to but also uptake, bioactivation, and detoxification of xenobiotics. Incorporated into molecular epidemiology, biomarkers might provide useful information on carcinogenic mechanisms. However, a prerequisite for sound human biomonitoring is detailed knowledge of competing metabolic pathways. Risk estimation based on results of animal experiments are valid only when species-specific differences in metabolism and disposition are recognized and taken into account. For NNK, conflicting metabolic results have been reported in rodents and primates, including human. Therefore, the metabolism and disposition of NNK were studied using rhesus monkeys as a nonhuman primate model.

Within 24 h after a single i.v. injection of 5 to 10 μg of [5-3H]NNK/kg, monkeys excreted >85% of radioactivity in urine (Table 1).

Hydroxy acid, the end product of NNAL α-hydroxylation, was the major metabolite (29% of the dose) followed by two isomeric NNAL glucuronides (20%), keto acid (14%) and NNAL (9%). These results indicate significant metabolic activation of NNK (46%) in accord with previous experiments on patas monkeys (Hecht et al., 1993b). Two major differences in low-dose NNK metabolism in primates compared with rats were confirmed. 1) Rats metabolize NNK more directly by α-hydroxylation to yield keto acid, the end product of NNK α-hydroxylation, whereas monkeys produce mainly hydroxy acid. In two different rat experiments, the ratio of keto acid to hydroxy acid in 24- to 48-h urine was 2.4 (Morse et al., 1990) and 2.0 (Richter and Tricker, 1994), respectively. In the 24-h urine sample of patas monkeys (Hecht et al., 1993b) and rhesus monkeys, this ratio was 0.5:0.6. This difference between rats and monkeys may be important in view of the results of Hecht’s group, which show that not only methylation from both NNK and NNAL methylene hydroxylation but also pyridoxobutylation, which can arise from methyl hydroxylation of NNK.
only, is involved in carcinogenesis and mutagenesis by NNK (Hecht, 1998b). At NNK doses in the lower microgram per kilogram range, rats excrete very low amounts of NNAL and/or NNAL-Gluc. Collectively, these metabolites accounted for only 6 to 7% of the dose in 48-h urine from F344 rats (Morse et al., 1990) and 24-h urine from Wistar rats (Richter and Tricker, 1994). In monkey urine, NNAL + NNAL-Gluc(I + II) made up 21% (Hecht et al., 1993b) and 32% (present result) of radioactivity in 24-h urine. It should be emphasized that excretion of these metabolites does not indicate NNK activation. Increased NNAL + NNAL-Gluc in the urine of smokers consuming watercress was taken as an indication of inhibition of NNK metabolic activation (Hecht et al., 1995). NNAL + NNAL-Gluc in the urine of smokers and nonsmokers (Carmella et al., 1993a; Hecht et al., 1993a; Meger et al., 1996; Parsons et al., 1998) are only weak biomarkers even if they are detectable because they cannot account for the total NNK uptake. They are definitely not biomarkers of NNK bioactivation.

Even 10 days after a single i.v. dose of 5 to 10 μg of [5-3H]NNK/kg, all major NNK metabolites could be determined in the urine of rhesus monkey urine (Fig. 2). The total amount of radioactivity in 24-h urine 10 days after NNK injection accounted for 0.2 ± 0.1%. This contrasts with results in rats in which no radioactivity above the background, i.e., less than 0.01% of the dose, could be detected in 24-h urine 3 days after a single s.c. dose of 17 μg of [1-14C]NNK/kg (Richter and Tricker, 1994).

Decay of total radioactivity and all major metabolites in the urine of rhesus monkeys could be described best by a two-compartment model with half-lives of about 1.5 h and 2 days (Table 1; Fig. 5). A similar initial half-life (0.5–1.5 h) has been reported for decay of NNK and NNAL in serum of baboons and patas monkeys (Adams et al., 1985; Hecht et al., 1993b). In the case of nicotine, the slow decay of radioactivity in urine of smokers as well as stump-tailed macaques could be explained by the much longer half-life of cotinine and other nicotine metabolites, which are in the range of 10 to 20 h compared with the short half-life of about 1 to 2 h for nicotine itself (Kyerematén et al., 1990; Seaton et al., 1991). The estimated half-lives for the urinary decay of nicotine and cotinine compare favorably with their half-lives in serum (Pérez-Stable et al., 1998). Although with time, a shift in favor of NNK detoxification products is observed in rhesus monkey urine (Fig. 4), the slow compartment of NNK disposition in urine cannot be explained by slower metabolism of any NNK metabolite (Table 1). Therefore, a deep compartment may exist, releasing NNK and/or NNAL over an extended period of several weeks. One possibility for such a compartment is melanin, which has been shown to accumulate high concentrations of radioactivity in NNK-treated rodents and marmosets (Castonguay et al., 1983, 1984, 1985; Tjälve and Castonguay, 1983). In marmosets, 4 h after NNK administration, >95% of the radioactivity in the eye was accounted for by NNAL. Melanin has the structure of a polyanion and binds electrostatically with basic compounds (Larsson and Tjälve, 1979). Differences between rats and rhesus monkeys in NNK disposition could be explained in part by the lack of substantial amounts of melanin in albino rats. Gerstenberg et al. (1995) demonstrated a 20-fold higher accumulation of systemically administered nicotine in hair of pigmented (Brown Norway) compared with albino rats (Sprague-Dawley).

The preference for the NNAL α-hydroxylation pathway in monkeys compared with rats may not fully explain the observed differences in urinary disposition of NNK metabolites. The present experiments clearly show that biliary excretion of NNAL-Gluc is quantitatively unimportant in rhesus monkeys compared with rats (Schulze et al., 1992). One reason for this could be the well known difference between rats and primates in molecular threshold for biliary excretion of xenobiotics (Hirom et al., 1977). Although considerable progress has been made in understanding mechanisms of biliary excretion, no explanation for these species differences can be offered presently (Yamazaki et al., 1996). However, differences between humans and monkeys and rodents exist in expression of multidrug resistance genes encoding P-glycoproteins, the putative carrier proteins involved in biliary excretion (Gant et al., 1995). Consequences are obvious differences between rats and primates in biliary excretion of NNAL-Gluc. In rats, NNAL-Gluc can undergo enterohepatic recycling, and its further metabolism can produce NNAL and/or NNK α-hydroxylation and N-oxidation (Atawodi et al., 1994). By contrast, in primates, NNAL-Gluc(I + II), once formed, would be excreted rapidly through the kidneys and, thus, cannot cause further harm.

Disposition of total radioactivity in plasma of one female rhesus monkey after i.v. injection of 28.4 μg/kg [5-3H]NNK was best described by a two-compartment model. No attempt was made to calculate kinetic parameters for individual NNK metabolites. Rapid disappearance of NNK reported for patas monkeys (Hecht et al., 1993b) is confirmed in the present experiment. The half-life of the second compartment of radioactivity decay in the rhesus monkey is 2 to 10 times longer than half-lives reported for patas monkeys. One reason for this discrepancy could be that in patas monkeys NNK elimination was measured in awake animals, whereas we used a single rhesus monkey in deep isoflurane anesthesia. As discussed in detail previously, the low dose of ketamine used for premedication is unlikely to have influenced NNK metabolism (Seaton et al., 1991). However, the long half-lives observed for urinary excretion in awake male rhesus monkeys indicate that a third compartment with much lower clearance should exist that could not be evaluated in the

### TABLE 3

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>% Distribution of Metabolites in Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>Hydroxy acid</td>
<td>8.9</td>
</tr>
<tr>
<td>Keto acid</td>
<td>8.7</td>
</tr>
<tr>
<td>NNAL-Gluc(I + II)</td>
<td>7.5</td>
</tr>
<tr>
<td>NNAL-N-oxide</td>
<td>2.7</td>
</tr>
<tr>
<td>Diol</td>
<td>N.D.</td>
</tr>
<tr>
<td>Keto alcohol</td>
<td>8.3</td>
</tr>
<tr>
<td>NNAL</td>
<td>28.1</td>
</tr>
<tr>
<td>NNK</td>
<td>35.9</td>
</tr>
<tr>
<td>Σ of α-hydroxylation&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.6</td>
</tr>
<tr>
<td>NNAL + NNAL-Gluc</td>
<td>35.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> One female rhesus monkey (5.2 kg) received i.v. 1.72 mCi of [5-3H]NNK (28.4 μg/kg). Blood was collected for 6 h, and plasma was analyzed by HPLC as described in Materials and Methods.

<sup>b</sup> Including hydroxy acid, keto acid, diol, and keto alcohol.

<sup>c</sup> Not detected (<1% of total radioactivity analyzed by HPLC).
short-term experiment in the female monkey. Therefore, the linear increase of NNK clearance with body weight of different species (Hecht et al., 1993b) most probably applies only for the rapid phase of NNK elimination.

Accumulation of NNK-derived radioactivity in the stomach probably can be ascribed to sequestration directly into the stomach lumen, as reported previously to occur after i.v. administration in rodents and marmosets (Castonguay et al., 1983, 1984, 1985; Tjälve and Castonguay, 1983). In marmosets, NNAL constituted 86% of the total radioactivity present in the stomach lumen 4 h after i.v. injection of NNK. Because we did not sample stomach contents completely, the total amount of NNAL excreted within 6 h into the stomach can be estimated only roughly as well below 1% of the dose.

In conclusion, metabolism and disposition of NNK in rhesus monkeys differ from those in rodents. With respect to urinary excretion of NNAL and NNAL-Gluc, humans behave more like rhesus monkeys than like rodents. These interspecies differences should be recognized and considered in attempting to extrapolate quantitatively results from tumor experiments in rodents to humans. Additional comparative studies on example-given carcinogenicity in monkeys and on NNK metabolism in vitro in rodent, monkey, and human tissues (Richter et al., 1998) will be needed to permit a more precise evaluation of health hazards imposed on humans by NNK.

References


