

Detection of Aminophenylnorharman, a Possible Endogenous Mutagenic and Carcinogenic Compound, in Human Urine Samples

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Abstract

Mutagenic/carcinogenic 9-(4'-aminophenyl)-9H-pyrido[3,4-b]indole [aminophenylnorharman (APNH)] is formed from norharman and aniline in the presence of cytochrome P450 3A4/1A2. Because both precursors are widely distributed in the environment, human exposure is unavoidable. To clarify APNH formation in the human body, amounts of the compound in 24-h human urine collected from smokers and nonsmokers, eating a normal diet, were analyzed by liquid chromatography/electrospray ionization tandem mass spectrometry. In addition, norharman and aniline were also analyzed by high-performance liquid chromatography and gas chromatography, respectively. APNH could be detected in all urine samples at levels 49 to 449 pg for smokers and 21 to 594 pg for nonsmokers per 24-h urine, respectively. The amounts of norharman and aniline were 46 to 185 ng

and 0.70 to 8.10 μ g for smokers and 52 to 447 ng and 0.49 to 5.72 μ g for nonsmokers, respectively, per 24-h urine (none of the levels differing significantly between smokers and nonsmokers). To exclude exogenous exposure to norharman and aniline, we analyzed the levels of APNH, norharman, and aniline in urine samples collected from inpatients receiving parenteral alimentation. Similar to the healthy volunteers, all urine samples contained 12 to 338 pg of APNH, 6 to 75 ng of norharman, and 0.33 to 1.86 μ g of aniline per 24-h urine. These results suggest that APNH should be considered as a novel endogenous mutagen/carcinogen; thus, it is very important to determine the biological significance of this carcinogen for human cancer development. (Cancer Epidemiol Biomarkers Prev 2007; 16(1):151-6)

Introduction

9H-pyrido[3,4-b]indole (norharman), widely distributed in our environment, such as in cigarette smoke and cooked foods (1), is not mutagenic to *Salmonella typhimurium* strains, either with or without S9 mix. However, it becomes mutagenic to *S. typhimurium* TA98 and YG1024 with S9 mix in the presence of nonmutagenic aromatic amines, such as aniline and *o*-toluidine (2-4). We have reported that the appearance of mutagenicity derived from norharman and aniline in the presence of S9 mix is due to the formation of a mutagenic compound 9-(4'-aminophenyl)-9H-pyrido[3,4-b]indole [aminophenylnorharman (APNH)], shown in Fig. 1 (5, 6). Moreover, it has been shown that enzymes mainly responsible for APNH formation from norharman and aniline are cytochrome P450 3A4 (CYP3A4) and CYP1A2 (7). APNH is thought to be metabolically converted to a hydroxyamino derivative by CYP1A2 and further activated to form esters by acetyltransferase, then covalently binding to DNA bases, with *N*⁴-(2'-deoxyguanosin-8-yl)-9-(4'-aminophenyl)-9H-pyrido[3,4-b]indole (dG-C8-APNH) as the main adduct (8). Mutagenic activity of APNH in *S. typhimurium* strains proved comparable

with those of 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and 3-amino-1-methyl-5H-pyrido[4,3-b] indole (Trp-P-2), food-borne carcinogenic heterocyclic amines (5, 9). In a long-term carcinogenicity experiment using F344 rats, APNH induced tumors in the liver, colon, thyroid, hematopoietic systems, and clitoral gland (10). To investigate the *in vivo* formation of APNH from norharman and aniline, we analyzed APNH in urine of rats and detected the compound only in animals fed norharman and aniline and not in the controls or with either norharman or aniline alone (11). Therefore, it is very likely that APNH is formed from norharman and aniline *in vivo*.

Norharman has been reported to be present at much higher levels than mutagenic and carcinogenic heterocyclic amines, such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and MeIQx, in cigarette smoke condensate and cooked foodstuffs and indeed was detected in human urine samples at levels much higher than for PhIP or MeIQx (12, 13). Moreover, aniline is not only used in various ways as an industrial raw material but also ubiquitously present in cigarette smoke condensate and some vegetables and has also been detected in human urine and breast milk (14-17). Thus, humans may be continuously and simultaneously exposed to norharman and aniline in daily life, raising the possibility of APNH production in our bodies. In the present study, we therefore examined this possibility using human urine samples, analyzing levels of APNH, norharman, and aniline. Because norharman and aniline are contained in cigarette smoke condensates, separate groups of smokers and nonsmokers were recruited. Furthermore, to assess the contribution of diet, we also obtained urine samples from inpatients receiving parenteral alimentation.

Received 1/24/06; revised 8/16/06; accepted 11/7/06.

Grant support: Ministry of Health, Labour and Welfare of Japan grants-in-aid for Cancer Research and for the Third-Term Comprehensive 10-Year Strategy for Cancer Control.

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doi:10.1158/1055-9965.EPI-06-0052

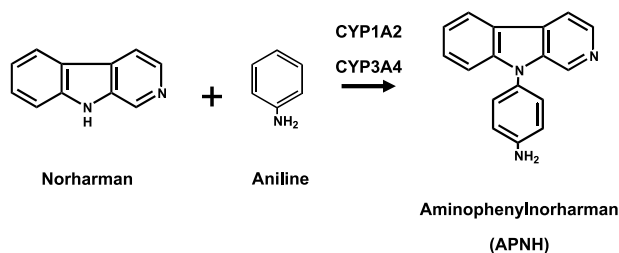


Figure 1. Formation of mutagenic/carcinogenic APNH by reaction of norharman with aniline in the presence of CYPs.

Materials and Methods

Chemicals and Reagents. Standard APNH and [$^2\text{H}_4$]APNH (>99.8% isotopically pure) were obtained from the Nard Institute (Osaka, Japan). Norharman hydrochloride and aniline were purchased from Katsura Chemical (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively, and *N*-methyl-*m*-toluidine and *n*-propyl chloroformate were from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals were of analytic reagent grade. Blue rayon, a product specially developed for binding and recovering polycyclic carcinogens, was purchased from Funakoshi (Tokyo, Japan).

Collection of Urine Samples. Data for the study population characteristics are summarized in Table 1. Urine samples were collected from healthy volunteers living in Japan: 16 males (22–54 years old) and 2 females (28 and 34 years old). During the experimental period, none of these volunteers had any dietary and smoking restrictions or took any medication. Nine of the male volunteers were smokers, consuming 9 to 25 common brand Japanese filtered cigarettes per day. Urine samples were also obtained from eight inpatients, receiving parenteral alimentation, including five males (45–67 years old) and three females (59–73 years old), undergoing operations for colorectal cancer at the National Cancer Center Hospital. During the period of collection of urine samples, none of patients ate any food or smoked. Collected 24-h urine samples were frozen and stored at -20°C until analyses. The study procedures were approved by the ethics committee of the National Cancer Center, Tokyo, Japan.

Partial Purification of Urine Samples. Samples of 50-mL urine were separated for analysis of aniline, and the remainder was used for the analysis for APNH and norharman. [$^2\text{H}_4$]APNH was used as an internal standard and added to

each urine sample before blue rayon treatment. The urine sample was treated with 6 g blue rayon for three times, which was then washed with water, and adsorbed materials were extracted with 1.2 liters of methanol/ammonia water (50:1, v/v), as previously reported (13). The extracts were evaporated to dryness, and the residues were dissolved in methanol and further purified by high-performance liquid chromatography (HPLC). The material was applied to an analytic-grade TSK gel octadecyl silane-80Ts column (5- μm particle size, 4.6 mm \times 250 mm; Tosoh, Tokyo, Japan) and eluted at a flow rate of 1 mL/min with the following system: 0 to 30 min, 15% acetonitrile in 25 mmol/L phosphate buffer (pH 2); 30 to 60 min, a linear gradient of 15% to 50% acetonitrile in 25 mmol/L phosphate buffer (pH 2). The fractions with retention times of 5 to 15 min and 30 to 40 min, corresponding to those of authentic norharman and APNH, respectively, were collected and evaporated to dryness. For further purification of APNH, the residue of the fraction corresponding to APNH was dissolved in 50% methanol and injected into the same column with a mobile phase of 45% acetonitrile in 0.05% of diethylamine adjusted to pH 6 with acetic acid. The fractions with retention times of 13 to 18 min, corresponding to authentic APNH, were collected and then analyzed by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS) as described below. All the above HPLC procedures were done at ambient temperature.

To examine the formation of APNH during the above partial purification process, authentic norharman (150 ng) and aniline (3000 ng), of which levels are almost equivalent to those detected in urine, were added to 2.0 liters of water. [$^2\text{H}_4$]APNH as an internal standard was also added to the water solution and treated with blue rayon, then purified by HPLC under the same conditions as described above.

Determination of APNH. The purified samples containing APNH were dissolved in 100 μL of 50% methanol, and aliquots were injected into a COSMOSIL 5C $_{18}$ -AR-II column (5- μm particle size, 2.0 mm \times 150 mm; Nacalai Tesque). The flow rate was 0.3 mL/min with the following system: 0 to 3 min, 5% acetonitrile in 0.1% formic acid; 3 to 10 min, a linear gradient of 5% to 95% acetonitrile in 0.1% formic acid. Detection and quantification of APNH were done by LC/ESI/MS/MS with a 4000 Q TRAP (Applied Biosystems, Foster City, CA). The total scan time, including the pause, was 1 s. The ion spray voltage was set at 5 kV, and the collision energy was set at 51 V with a source temperature of 600 $^\circ\text{C}$, using N_2 as the collision gas.

The analysis conditions used to fragments of APNH and [$^2\text{H}_4$]APNH were each optimized for maximum sensitivity for the variables analyzed.

Table 1. Amounts of APNH, norharman, and aniline in urine samples from healthy volunteers

Subject	Age (y)	Sex	Cigarettes per day	APNH (pg/24-h urine)	Norharman (ng/24-h urine)	Aniline (μg /24-h urine)
1	22	Male	0	158	149	5.72
2	22	Male	0	39	134	3.59
3	23	Male	9	86	86	1.26
4	23	Male	9	86	115	0.70
5	23	Male	10	443	108	6.34
6	23	Male	10	242	95	4.26
7	24	Male	10	49	91	5.01
8	28	Female	0	111	113	4.36
9	29	Male	20	449	109	1.01
10	30	Male	0	594	285	1.09
11	30	Male	15	102	185	2.86
12	31	Male	0	203	324	2.64
13	34	Female	0	56	181	0.92
14	35	Male	0	24	52	3.07
15	35	Male	25	101	46	1.41
16	44	Male	0	244	447	2.55
17	44	Male	20	195	61	8.10
18	54	Male	0	21	64	0.49

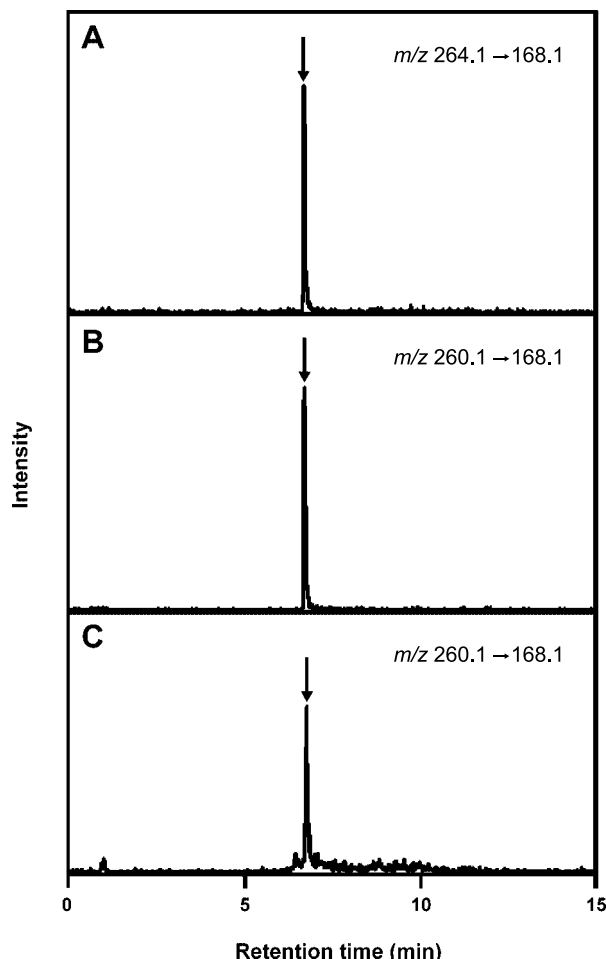


Figure 2. Typical LC/ESI/MS/MS chromatograms of authentic APNH and human urine sample in the multiple reaction monitoring mode. Product ion mass spectra (A) at m/z 264.1 \rightarrow 168.1 for $[^2\text{H}_4]$ APNH as an internal standard and (B) at m/z 260.1 \rightarrow 168.1 for authentic APNH and (C) a human sample from a healthy volunteer (subject 8). Peaks corresponding to APNH are indicated by arrows.

Alternating scans were employed to isolate $[\text{M} + \text{H}]^+$ ions at masses of 260.1 for APNH and 264.1 for $[^2\text{H}_4]$ APNH. For quantitative measurements, the multiple reaction monitoring transitions used for APNH and $[^2\text{H}_4]$ APNH were major fragment ions m/z 260.1 \rightarrow 168.1 and m/z 264.1 \rightarrow 168.1, respectively. When authentic APNH and $[^2\text{H}_4]$ APNH were analyzed under these conditions, both single peaks exhibiting fragment ions at m/z 260.1 \rightarrow 168.1 and m/z 264.1 \rightarrow 168.1 were eluted at a retention time of 6.8 min, and the detection limit with 24-h urine samples was 0.15 pg.

Determination of Norharman. Analysis of norharman was done using the same octadecyl silane column, by an FS-8011 fluorometric detector (Tosoh) with excitation and emission wavelengths of 260 nm and 430 nm, respectively. When a mobile phase of 30% acetonitrile in 0.05% of diethylamine adjusted to pH 6 with acetic acid was pumped in at a flow rate of 1 mL/min, norharman was clearly seen as a single peak at a retention time of 17 min. Under these conditions, the detection limit with 24-h urine samples was 1.0 pg. The recovery of norharman during the purification process was estimated by spiking with an equivalent level of authentic norharman to that detected in urine, and the value was found to be 80%.

To confirm the mass spectra, all samples were analyzed using a Waters ZQ2000 mass spectrometer (Waters Corp., Milford, MA) equipped with an Agilent 1100 HPLC system

(Agilent Technologies, Santa Clara, CA) with an analytic-grade Wakosil-II3C18HG column (3- μm particle size, 2.0 mm \times 150 mm; Wako Pure Chemical, Osaka, Japan). Elution was with a flow rate of 0.3 mL/min with 30% acetonitrile in 0.05% of diethylamine adjusted to pH 6 with acetic acid. The LC/MS conditions used were as follows: ESI, positive mode, and single ion monitoring with m/z 169 for norharman. MS conditions were as follows: ion source temperature, 110°C; cone voltage, 50 eV.

Determination of Aniline. A sample of 50 ng of *N*-methyl-*m*-toluidine was added to 25 mL of urine as an internal standard, and after saturation with sodium chloride, the mixtures were extracted twice with 20 mL of *n*-hexane. After the *n*-hexane layer was collected, the combined extracts were back-extracted with 2.5 mL of 2 mol/L hydrochloric acid, and the aqueous layer was collected and washed with 2.5 mL of *n*-pentane, neutralized with 2 mol/L sodium hydroxide, and used as the sample for derivatization.

To the sample solution was added 0.25 mL of 10% sodium carbonate and 20 μL of *n*-propyl chloroformate, and the reaction mixture was saturated with sodium chloride, extracted twice with 0.5 mL of *n*-pentane, and evaporated. The residue was dissolved in 40 μL of *n*-hexane, and 1 or 2 μL of this solution was injected into a gas chromatograph by the hot-needle injection technique (needle dwell time, 3 s).

Gas chromatography analysis was carried out with an Agilent 6890 Series gas chromatograph equipped with a split/splitless capillary inlet system and a nitrogen-phosphorus detector and a fused-silica capillary column of cross-linked HP-5 (30 m \times 0.32 mm inner diameter, 0.25- μm film thickness; J&W, Folsom, CA) was used. The operating conditions were

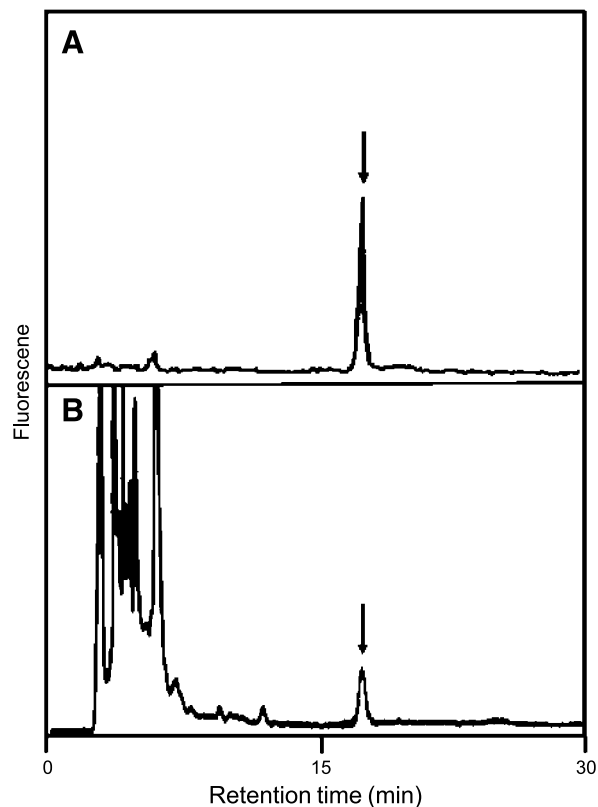


Figure 3. HPLC chromatograms obtained with authentic norharman and human urine sample. Authentic norharman (A) and a human urine sample (B; subject 8) were monitored for fluorescence with excitation and emission wavelengths of 260 and 430 nm, respectively. Peaks corresponding to the retention time of authentic norharman on the analytic octadecyl silane columns are indicated by arrows.

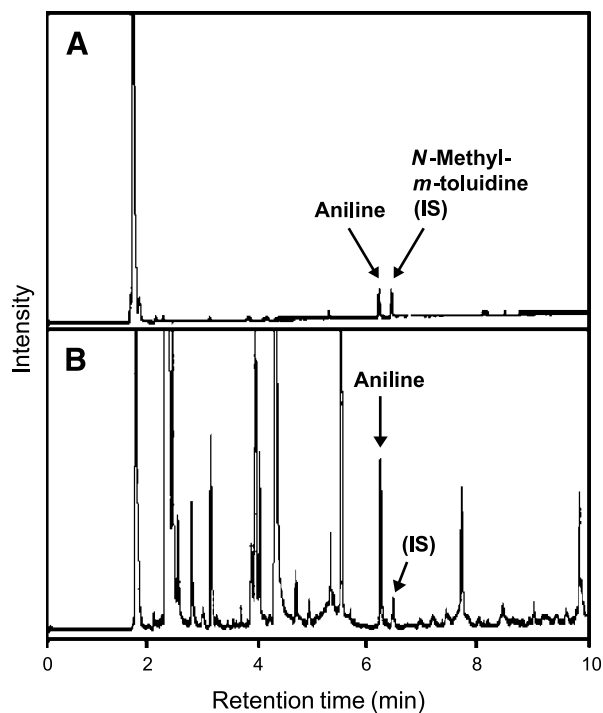


Figure 4. Gas chromatograms obtained with authentic norharman and human urine sample. Authentic aniline (A) and a human urine sample (B; subject 8) were analyzed by gas chromatography/nitrogen-phosphorus detector after derivatization with *n*-propyl chloroformate. Peaks corresponding to the *N*-propoxycarbonyl aniline derivative of aniline are indicated by arrows. *N*-methyl-*m*-toluidine was used as an internal standard (IS).

column temperature, programmed at 10°C/min from 120°C to 220°C; injection temperature, 270°C; detector temperature, 300°C; inlet helium flow rate, 1.5 mL/min; make-up gas flow rate, 30 mL/min; and split ratio, 10:1. Under these conditions, the detection limit with 24-h urine samples was 1.0 ng.

To confirm the compound in the peak fraction as aniline, a Shimadzu QP-5000 gas chromatograph-mass spectrometer system (Shimadzu Corp., Kyoto, Japan) was employed. The same eluates were analyzed with a single ion monitoring set at m/z 179 for *N*-propoxycarbonyl aniline and 93 for aniline under the same gas chromatography conditions. MS conditions were as follows: ion source temperature, 200°C; ionization voltage, 70 eV.

Results

Typical chromatograms of LC/ESI/MS/MS analyses of 1 pg of standard [$^2\text{H}_4$]APNH and APNH are presented in Fig. 2A and B, respectively. With LC/ESI/MS/MS using positive ESI and multiple reaction monitoring, two daughter fragment ions for APNH could be observed at m/z 93 [$\text{M} + \text{H} - \text{C}_{11}\text{H}_8\text{N}_2$] $^+$, attributed to loss of norharman, and m/z 168 [$\text{M} + \text{H} - \text{C}_6\text{H}_7\text{N}$] $^+$, attributed to loss of aniline. Although the signal intensity of m/z 93 was weak, the primary molecular weight transition (i.e., parent to primary daughter ion) was m/z 260.1 \rightarrow 168.1. Similarly, two daughter fragment ions for a tetradeuterated derivative at m/z 97 [$\text{M} + \text{H} - \text{C}_{11}\text{H}_8\text{N}_2$] $^+$ and m/z 168 [$\text{M} + \text{H} - \text{C}_6\text{H}_3\text{D}_4\text{N}$] $^+$, attributed to loss of norharman and aniline, respectively, exhibited primary molecular weight transitions of m/z 264.1 \rightarrow 168.1. Figure 2C shows a chromatogram for a urine sample containing APNH from a nonsmoker (subject 8). Secondary molecular weight transitions of m/z 260.1 \rightarrow 93.1, attributed to loss of norharman, were also

observed at the same retention time of m/z 260.1 \rightarrow 168.1 in the urine samples (data not shown). When another batch from the same subjects was analyzed, similar results were obtained. Moreover, the fragmentation patterns of the compound detected in the urine samples were confirmed by LC/ESI/MS/MS with enhanced product ion scanning (data not shown). The calculated amounts of APNH in the urine samples from healthy volunteers are summarized in Table 1. APNH could be detected in all of the urine samples analyzed. In those from the nine volunteers who were current smokers, the levels were 49 to 449 pg, and in nonsmokers, the levels were 21 to 594 pg.

With HPLC analysis, a single peak for norharman was clearly seen at a retention time of 16 min in all urine samples. The compound detected by HPLC in human urine samples was confirmed to be norharman from its mass spectrum data obtained by LC/MS analysis. A typical chromatogram of norharman in urine from a nonsmoker (subject 8) on an analytic octadecyl silane column is shown in Fig. 3. By correcting the amounts of norharman for its recovery, the levels in urine samples were calculated, as shown in Table 1. Values for 24-h urine samples were 46 to 185 ng for smokers and 52 to 447 ng for nonsmokers.

A peak for aniline was also detected, by gas chromatography/nitrogen-phosphorus detector, in all urine samples, and a typical gas chromatography pattern with urine from a nonsmoker (subject 8) is shown in Fig. 4. The levels in 24-h urine samples were 0.70 to 8.10 μg for smokers and 0.49 to 5.72 μg for nonsmokers. In addition, the compound was confirmed to be aniline by gas chromatography/MS analysis.

Furthermore, APNH, norharman, and aniline were also detected in all urine samples from inpatients receiving parenteral alimentation (subjects a-h; see Table 2), at 12 to 338 pg, 6 to 75 ng, and 0.33 to 1.86 μg , respectively.

On the other hand, APNH was not detected in the water solution containing norharman and aniline, after treatment with blue rayon. The level of APNH in the solution must be <0.15 pg, if present. This result suggests that APNH could not be formed from norharman and aniline during the extraction procedure with blue rayon.

Discussion

This is the first demonstration that mutagenic/carcinogenic APNH is detectable in human urine, all of the 24-h urine samples from 18 healthy volunteers, both of smokers and nonsmokers, and eating an ordinary diet being found positive. While we expected levels of APNH in urine from smokers to be much higher than those of nonsmokers, given the presence of norharman and aniline in tobacco smoke (1, 17), no significant difference was in fact observed (195 \pm 154 pg versus 161 \pm 181 pg, respectively). Comparison of norharman and aniline levels in human urine samples between smokers and nonsmokers also showed no significant differences. Thus,

Table 2. Amounts of APNH, norharman, and aniline in urine samples from patients

Subject	Age (y)	Sex	APNH (pg/24-h urine)	Norharman (ng/24-h urine)	Aniline (μg /24-h urine)
a	45	Male	13	30	1.86
b	58	Male	39	6	1.16
c	59	Female	86	75	1.68
d	60	Female	18	7	0.33
e	60	Male	14	13	0.60
f	60	Male	339	19	1.79
g	67	Male	13	9	1.06
h	73	Female	165	39	1.05

NOTE: For patients receiving parenteral alimentation.

it is suggested that factors other than cigarette smoking, such as diet and endogenous formation, might greatly contribute to urinary levels of APNH, norharman, and aniline.

Therefore, we analyzed the amounts of APNH, norharman, and aniline in human urine samples from inpatients who were not eating an ordinary diet or smoking. Surprisingly, all of the urine samples were again found to contain not only APNH but also norharman and aniline; although the level of APNH did seem to be lower than with the healthy volunteers, the urinary levels of norharman and aniline were significantly lower. However, as there is a significant imbalance with age and gender among the series of samples, further studies are required for the evaluation of APNH exposure levels in each of the groups.

It has been reported that norharman is produced by pyrolysis of tryptophan (18) and is present at high levels in cigarette smoke condensate and various kinds of cooked foods (1). Moreover, norharman is known to be formed endogenously and be detectable in human urine samples from both healthy volunteers eating an ordinary diet and patients receiving parenteral alimentation (12, 19). Aniline is also reported to be present at high levels in our environment, such as in cigarette smoke and some vegetables, and is detected in human urine samples from healthy volunteers (14, 15, 17). However, in this study, aniline could be detected in human urine samples not only from healthy volunteers but also from patients receiving parenteral alimentation. Because aniline did not exist at detectable levels in the intravenous preparation used for parenteral alimentation, it is suggested that aniline as well as norharman might be formed endogenously (data not shown). At present, the precursors and the mechanisms for the formation of aniline in human bodies are unclear, and further studies, including aniline biosynthesis, are needed.

In the present study, we only analyzed unchanged forms of APNH, norharman, and aniline in human urine samples from healthy volunteers and patients. It has been reported that other food-derived heterocyclic amines, such as PhIP and MeIQx, are metabolized to glucuronate and sulfate conjugates and then excreted in urine. Moreover, we have reported an increase in the amount of APNH in urine samples from rats given norharman and aniline treated with 1 N hydrochloric acid (11). The results suggested that more than half of the APNH produced *in vivo* from norharman and aniline might be excreted into urine as conjugated forms, so that APNH might also exist as conjugates, such as glucuronates, in human bodies. It has been reported that UDP-glucuronosyltransferase expression is modulated by environmental factors, including diet and hormones (20-22). Although this has yet to be clarified, the UDP-glucuronosyltransferase expression levels in healthy volunteers and patients might differ and explain the comparable levels of unchanged APNH, despite a greater likely exposure in the healthy volunteers. On the other hand, we have recently reported that CYP1A2 and CYP3A4 mainly contributed to formation of APNH from norharman and aniline (7). As in the case of the phase II enzyme UDP-glucuronosyltransferase, these phase I enzymes show interindividual variation in expression caused by environmental factors and polymorphisms (23, 24). This would explain the wide range of values for APNH formation detected in this study.

We have previously reported that norharman is mutagenic to *S. typhimurium* TA98 with S9 mix in the presence of *o*-toluidine, an aromatic amine, as well as aniline (2-4). In addition, another β -carboline compound (harman) shows comutagenicity with aromatic amines in the presence of S9 mix (4). The appearance of mutagenicity was caused by the formation of various mutagenic compounds [i.e., aminophenyl- β -carbolines, such as 9-(4-amino-3'-methylphenyl)-9H-pyrido[3,4-*b*]indole, 9-(4-amino-3'-methylphenyl)-1-methyl-9H-pyrido[3,4-*b*]indole, and

9-(4'-aminophenyl)-1-methyl-9H-pyrido[3,4-*b*]indole (25, 26)]. Because harman and *o*-toluidine are also widely distributed in the environment (1, 17, 27), humans could be continuously exposed, and aminophenyl- β -carbolines are also probably formed in the human body.

In conclusion, we here detected APNH in human urine samples from both healthy volunteers and inpatients. Because this did not seem to be dependent on dietary intake or cigarette smoking, the compound can be considered a new type of endogenous mutagen/carcinogen. The level of APNH in human urine samples was much lower than found earlier for MeIQx, but comparable with that for 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1; ref. 13). However, APNH is a more potent carcinogen than Trp-P-1 and MeIQx (10, 28), so that the risk of human cancer might be similar. To understand the role of APNH in human cancer development, studies for APNH levels in human urine samples, which are balanced by age and gender, APNH metabolism, and other factors that affect APNH levels, will be required.

Acknowledgments

We thank Drs. Yuji Haishima and Toshie Tsuchiya (Division of Medical Devices, NIH Sciences) for providing the measuring instruments.

References

1. Totsuka Y, Ushiyama H, Ishihara J, et al. Quantification of the co-mutagenic beta-carbolines, norharman and harman, in cigarette smoke condensates and cooked foods. *Cancer Lett* 1999;143:139-43.
2. Nagao M, Yahagi T, Honda M, Seino Y, Matsushima T, Sugimura T. Demonstration of mutagenicity of aniline and *o*-toluidine by norharman. *Proc Jpn Acad* 1977;53B:34-7.
3. Nagao M, Yahagi T, Sugimura T. Differences in effects of norharman with various classes of chemical mutagens and amounts of S-9. *Biochem Biophys Res Commun* 1978;83:373-8.
4. Sugimura T, Nagao M, Wakabayashi K. Metabolic aspects of comutagenic action of norharman. In: Snyder R, Jollow DJ, Parke DV, Gibson CG, Kocsis JJ, Witmer CM, editors. *Biological reactive intermediates-II. Chemical mechanisms and biological effects: Part B*. New York: Plenum Press; 1982. p. 1011-25.
5. Totsuka Y, Hada N, Matsumoto K, et al. Structural determination of a mutagenic aminophenylnorharman produced by the co-mutagen norharman with aniline. *Carcinogenesis* 1998;19:1995-2000.
6. Sugimura T. A new concept of co-mutagenicity from a phenomenon forgotten for the past two decades: is it more important than previously expected? *Environ Health Perspect* 1998;106:A522-3.
7. Nishigaki R, Totsuka Y, Takamura-Enya T, Sugimura T, Wakabayashi K. Identification of cytochrome P-450s involved in the formation of APNH from norharman with aniline. *Mutat Res* 2004;562:19-25.
8. Totsuka Y, Takamura-Enya T, Kawahara N, Nishigaki R, Sugimura T, Wakabayashi K. Structure of DNA adduct formed with aminophenylnorharman, being responsible for the comutagenic action of norharman with aniline. *Chem Res Toxicol* 2002;15:1288-94.
9. Wakabayashi K, Nagao M, Esumi H, Sugimura T. Food-derived mutagens and carcinogens. *Cancer Res* 1992;52:2092-8s.
10. Kawamori T, Totsuka Y, Uchiya N, et al. Carcinogenicity of aminophenyl-norharman, a possible novel endogenous mutagen, formed from norharman and aniline, in F344 rats. *Carcinogenesis* 2004;25:1967-72.
11. Totsuka Y, Kataoka H, Takamura-Enya T, Sugimura T, Wakabayashi K. *In vitro* and *in vivo* formation of aminophenylnorharman from norharman and aniline. *Mutat Res* 2002;506-507:49-54.
12. Ushiyama H, Oguri A, Totsuka Y, Itoh H, Sugimura T, Wakabayashi K. Norharman and harman in human urine. *Proc Jpn Acad* 1995;71B: 57-60.
13. Ushiyama H, Wakabayashi K, Hirose M, Itoh H, Sugimura T, Nagao M. Presence of carcinogenic heterocyclic amines in urine of healthy volunteers eating normal diet, but not of inpatients receiving parenteral alimentation. *Carcinogenesis* 1991;12:1417-22.
14. IARC. Aniline and Aniline Hydrochloride. IARC monographs on the evaluation of carcinogenic risks of chemicals to humans. Lyon: IARC; 1982. p. 39-61.
15. Riffelmann M, Muller G, Schmieding W, Popp W, Norpoth K. Biomonitoring of urinary aromatic amines and arylamine hemoglobin adducts in exposed workers and nonexposed control persons. *Int Arch Occup Environ Health* 1995;68:36-43.
16. DeBruin LS, Pawliszyn JB, Josephy PD. Detection of monocyclic aromatic amines, possible mammary carcinogens, in human milk. *Chem Res Toxicol* 1999;12:78-82.
17. Luceri F, Pieraccini G, Moneti G, Dolara P. Primary aromatic amines from side-stream cigarette smoke are common contaminants of indoor air. *Toxicol Ind Health* 1993;9:405-13.

18. Sugimura T, Kawachi T, Nagao M, et al. Mutagenic principle(s) in tryptophan and phenylalanine pyrolysis products. *Proc Jpn Acad* 1997;53: 58–61.
19. Fekkes D, Tuiten A, Bom I, Pepplinkhuizen L. Tryptophan: a precursor for the endogenous synthesis of norharman in man. *Neurosci Lett* 2001;303: 145–8.
20. Walters DG, Young PJ, Agus C, et al. Cruciferous vegetable consumption alters the metabolism of the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in humans. *Carcinogenesis* 2004;25: 1659–69.
21. Xie W, Yeuh MF, Radomska-Pandya A, et al. Control of steroid, heme, and carcinogen metabolism by nuclear pregnane \times receptor and constitutive androstane receptor. *Proc Natl Acad Sci U S A* 2003;100:4150–5.
22. Li YQ, Prentice DA, Howard ML, Mashford ML, Desmond PV. The effect of hormones on the expression of five isoforms of UDP-glucuronosyltransferase in primary cultures of rat hepatocytes. *Pharm Res* 1999;16:191–7.
23. Xu C, Li CY, Kong AN. Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res* 2005;28:249–68.
24. Nagata K, Yamazoe Y. Genetic polymorphism of human cytochrome p450 involved in drug metabolism. *Drug Metab Pharmacokinet* 2002;17:167–89.
25. Hada N, Totsuka Y, Enya T, et al. Structures of mutagens produced by the co-mutagen norharman with *o*- and *m*-toluidine isomers. *Mutat Res* 2001;493:115–26.
26. Totsuka Y, Takamura-Enya T, Nishigaki R, Sugimura T, Wakabayashi K. Mutagens formed from beta-carbolines with aromatic amines. *J Chromatogr B* 2004;802:135–41.
27. IARC. *Ortho*-toluidine and *Ortho*-toluidine hydrochloride. IARC monographs on the evaluation of carcinogenic risks of chemicals to humans. Lyon: IARC; 1982. p. 155–75.
28. Sugimura T, Wakabayashi K, Nakagama H, Nagao M. Heterocyclic amines: mutagens/carcinogens produced during cooking of meat and fish. *Cancer Sci* 2004;95:290–9.

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Cancer Epidemiol Biomarkers Prev 2007;16:151-156.

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