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Mass Spectrometric Analysis of Tobacco-specific Nitrosamine Hemoglobin Adducts in Snuff Dippers, Smokers, and Nonsmokers[1]

Steven G. Carmella, Shashi S. Kagan, Mark Kagan, Peter G. Foiles, George Palladino, Arthur M. Quart, Elaine Quart, and Stephen S. Hecht[2]

American Health Foundation, Valhalla, New York 10595 [S. G. C., S. S. K., M. K., P. G. F., E. Q., S. S. H.]; Chemistry Department, University of Pennsylvania, Philadelphia, Pennsylvania 19104 [G. P.]; and School of Dentistry, Columbia University, New York, New York [A. M. Q.]

ABSTRACT

Hemoglobin adducts of the carcinogenic tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosonornicotine were quantified in blood samples collected from snuff dippers, smokers, and nonsmokers. Mild base treatment of hemoglobin adducted by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone or *N'*-nitrosonornicotine releases 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB). HPB was enriched by solvent partitioning and derivatized to its pentafluorobenzoate. After purification by high performance liquid chromatography, HPB-pentafluorobenzoate was analyzed by capillary column gas chromatography with detection by negative ion chemical ionization mass spectrometry and selected ion monitoring. [4,4-D_2]HPB was used as internal standard. The detection limit for HPB-pentafluorobenzoate was approximately 100 amol/injection or 5 fmol/g hemoglobin. Mean adduct levels (fmol HPB/g hemoglobin) were 517 ± 538 (SD) in snuff dippers, 79.6 ± 189 in smokers, and 29.3 ± 25.9 in nonsmokers. Adduct levels in snuff dippers and in a subgroup of smokers were higher than would have been predicted solely based on estimates of exposure to tobacco-specific nitrosamines. The results of this study provide the first measurements of tobacco-specific nitrosamine hemoglobin adducts in humans and suggest new approaches to understanding the metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosonornicotine in humans.

INTRODUCTION

This paper describes the development of a sensitive mass spectrometry method for determining amounts of NNK[3] and NNN hemoglobin adducts in humans. The method was applied to blood samples obtained from snuff dippers, smokers, and nonsmokers. Paper 2 in this series describes the relationship between hemoglobin adducts, DNA adducts, and dose of NNK administered to rats (1). Paper 3 reports experiments designed to determine the role of cysteine of hemoglobin as a binding site for electrophiles generated in the metabolism of NNK (2).

We have proposed that the tobacco-specific nitrosamines NNK and NNN are among the important causative compounds for cancers associated with tobacco use (3–5). This proposal is based on their carcinogenic activities and concentrations in tobacco products. NNK is highly carcinogenic to the rodent lung, inducing adenocarcinoma and squamous cell carcinoma in rats, hamsters, or mice independent of the route of administration. NNK and its metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol are the only tobacco smoke constituents known to induce exocrine pancreatic tumors in laboratory animals (6). NNK also produces tumors of the liver and nasal cavity in rats. Among tobacco smoke constituents, NNN is the most abundant esophageal carcinogen. It also causes nasal cavity tumors in rats. A mixture of NNK and NNN swabbed in the rat oral cavity produced tumors of the cheek, tongue, and hard palate (7). NNK and NNN occur in ppm concentrations in unburned tobacco such as that used for snuff dipping and in the mainstream and sidestream smoke of cigarettes, generally in concentrations between 0.1 and 1 μg/cigarette. These data support our hypothesis that NNK and NNN are causative agents for oral cavity cancer in snuff dippers and for cancers of the lung, pancreas, and esophagus in smokers. However, limited data are available on the levels of tobacco-specific nitrosamines or their metabolites in humans. Tobacco-specific nitrosamines have been detected in the saliva of snuff dippers and betel quid chewers (8–11). Tobacco-specific nitrosamine hemoglobin adducts, as measured in this study, can be used as dosimeters for further evaluating the role of NNK and NNN in tobacco-related cancer.

Although the dose of NNK and NNN experienced by a snuff dipper or smoker could be estimated from the measured levels of these compounds in tobacco and tobacco smoke, these estimates have several uncertainties. The amounts of NNK and NNN extracted from tobacco by a snuff dipper or chewer, or the amounts inhaled by a smoker, depend on individual characteristics which would not necessarily be reflected under standard conditions used for determining levels of these compounds in tobacco or tobacco smoke. For example, it is known that smokers compensate in their smoking patterns in order to reach a certain dose of nicotine (12). NNK and NNN could also be formed endogenously in snuff dippers or smokers by reaction of nitrite or nitrogen oxides with nicotine or nornicotine (13–16). The endogenous formation of *N*-nitrosoproline has been repeatedly demonstrated in smokers (17–19). NNK and NNN require metabolic activation for binding to DNA, and the extents of these activation steps have been shown to vary widely in cultured human tissues (20). Modulation by other constituents of tobacco or tobacco smoke of enzyme activities involved in the metabolic activation of NNK and NNN would also affect their binding to cellular macromolecules. Thus, a method is needed to bypass these uncertainties and allow measurement of the dose of metabolically activated carcinogen which reaches target cellular macromolecules such as DNA. Hemoglobin adducts have been proposed as internal dosimeters for carcinogens (21). Advantages of hemoglobin adduct measurements include the ease of isolation of mg quantities of hemoglobin from blood and the long lifetime of the erythrocyte (120 days in humans) which allows estimation of chronic exposure. Hemoglobin adducts could be surrogates for DNA adducts, which are more difficult to measure in humans (22).

In a previous study, we showed that NNK and NNN form hemoglobin adducts in rats (23). The keto alcohol HPB is released upon mild base treatment of the adducted hemoglobin, as illustrated in Fig. 1. The structure of the hemoglobin adduct

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[2] To whom requests for reprints should be addressed, at American Health Foundation, 1 Dana Road, Valhalla, NY 10595.

[3] The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N'*-nitrosonornicotine; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; HPLC, high performance liquid chromatography; GC-NICI-MS, combined gas chromatography-negative ion chemical ionization mass spectrometry; i.d., inside diameter.

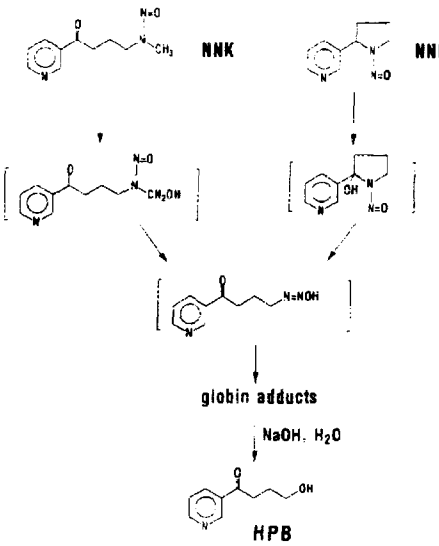

Fig. 1. Metabolic activation of NNK and NNN to intermediates which bind to globin and release HPB upon base hydrolysis.

which releases HPB is not known but it is believed to form by reaction with hemoglobin of the diazohydroxide illustrated in Fig. 1. The relationships of HPB formation to dose of NNK or NNN, as described in our previous study and in Ref. 1, indicate that it is a suitable dosimeter for exposure to these nitrosamines. Its facile release from hemoglobin by base treatment suggested that a sensitive method could be developed for its detection in humans. The method presented here is conceptually similar to those previously described for quantitation of hemoglobin adducts of ethylene oxide and 4-aminobiphenyl (24–26).

MATERIALS AND METHODS

Chemicals. HPB was synthesized (27). [5-^{3}H]NNK was obtained from Chemsyn Science Laboratories, Lenexa, KS. [4,4-D_2]HPB was prepared by *in vitro* metabolism of [4,4-D_2]NNK with microsomes from livers of rats pretreated with Aroclor 1254, as described previously (28, 29). It was isolated and purified by reverse phase HPLC (30). Standard HPB-pentafluorobenzoate was prepared under conditions similar to those described below. It was purified by silica gel thin layer chromatography, with elution by 10% $CHCl_3$ in methanol, and finally by reverse phase HPLC; NICI-MS 358.9 (100, M^-), 210.9 (5, $C_6F_5CO_2^-$), 166.9 (10, $C_6F_5^-$). [4,4-D_2]HPB-*pentafluorobenzoate* was prepared in a similar fashion. NICI-MS 360.9 [100, M^-], 359.9 [18, $(M-1)^-$], 210.9 [7, $C_6F_5CO_2^-$], 166.9 [13, $C_6F_5^-$]. HPB-tetrafluorobenzoate was prepared by reaction of HPB with 2,3,4,5-tetrafluorobenzoyl chloride, which was synthesized by reacting 2,3,4,5-tetrafluorobenzoic acid (Aldrich Chemical Co., Milwaukee, WI) with oxalyl chloride. HPB-tetrafluorobenzoate was purified by thin layer chromatography and reverse phase HPLC; NICI-MS 340.9 (100, M^-), 192.9 (60, $C_6HF_4CO_2^-$).

Instrumentation. MS analyses were performed with a Hewlett-Packard Model 5988A instrument. HPLC was carried out with a system consisting of two Model 510 pumps, a Model 680 automated gradient controller, a Model 440 fixed wavelength UV/visible detector, all from Waters Division of Millipore (Milford, MA), and a Model 7125 injector from Rheodyne (Cotati, CA).

Precautions to Minimize Contamination and Low Recovery in the Assay of Human Hemoglobin for HPB. As indicated below, some glassware was silanized by treatment with 5% dimethyldichlorosilane in toluene at room temperature for 1 h. It was then rinsed twice with toluene, three times with methanol, once with CH_2Cl_2, and twice with H_2O. All other glassware was rinsed several times with 95% CH_2Cl_2 in methanol. Centrifuge tubes and 4-ml vials had Teflon-capped liners; 20-ml vials had polyethylene cone liners. All equipment was in a dedicated laboratory used only for this assay. All organic solvents were reagent grade and were used without further purification. Aliquots of solvent necessary for each assay were poured into glass bottles with Teflon-lined caps. A 1 N NaOH solution was prepared from NaOH pellets and H_2O obtained from a Milli-Q H_2O purification system, Millipore (Bedford, MA). Reagent grade concentrated HCl was diluted with purified H_2O to 1 N and stored in glass bottles.

Collection of Blood and Isolation of Hemoglobin. Blood was collected in 10-ml heparinized Vacutainers (American Scientific Products, McGaw Park, IL): snuff dippers, four tubes; smokers and nonsmokers, one tube. The blood was centrifuged in a 38-ml plastic centrifuge tube at 900 × *g* for 10 min at 4°C. The supernatant was discarded and the packed RBC were washed three times by centrifugation with 3 volumes of 0.9% saline. The RBC were lysed by mixing with 3 volumes of ice cold H_2O on a vortex mixer (American Scientific Products) for 5 min. After the mixture stood for 15 min at 0°C, 0.67 M KH_2PO_4 buffer, pH 6.5, was added such that the final concentration was 0.2 M. The resulting mixture was centrifuged at 25,000 × *g* for 30 min at 4°C. The supernatant was removed and dialyzed overnight against 10–20 volumes of distilled H_2O, using Spectra/por 4 cellulose dialysis tubing, with a molecular weight cutoff of 12,000–14,000 (American Scientific Products). The dialysis H_2O was changed after 12 h and the sample was dialyzed again. The hemoglobin solutions were frozen at −78°C until analysis. Hemoglobin content was determined with Drabkin's reagent (Sigma Chemical Co., St. Louis, MO).

Analysis of Human Hemoglobin for HPB. Hemoglobin solution (5–8 ml), corresponding to 4–6 ml of blood, was added to a 50-ml disposable borosilicate centrifuge tube (Kimble, Toledo, OH) with a Teflon-lined cap. The normality was adjusted to 0.15 N with 1 N NaOH. The solution was sonically dispersed for 1 h at room temperature. The pH of the solution was then adjusted to 6–7 by addition of 1 N HCl. The pH was determined by withdrawing 1 μl with a 1-μl capillary pipet and applying this to pH paper. Globin precipitates in this step. A solution of [4,4-D_2]HPB (150 fmol) in 10 μl H_2O was added. The mixture was centrifuged at 2000–3000 rpm for 10 min at room temperature. The supernatant was transferred with a Pasteur pipet to another 50-ml centrifuge tube. The pH of the solution was adjusted to 1.5–2.5 by addition of 1 N HCl. It was extracted twice with equal volumes of CH_2Cl_2 and twice with equal volumes of hexane. If emulsions formed, they were broken by centrifugation at approximately 3000 rpm for 10 min. The organic extracts were discarded. The pH of the aqueous portion was adjusted to 6–7 with 1 N NaOH, and it was extracted three times with equal volumes of CH_2Cl_2. The combined CH_2Cl_2 layers were transferred with a Pasteur pipet to a silanized 50-ml centrifuge tube. The CH_2Cl_2 was removed using a Model SVC200H SpeedVac centrifugal concentrator (Savant Instruments, Farmingdale, NY). The residue was taken up in five approximately 200-μl aliquots of methanol. The combined methanol extracts were placed in a 4-ml screw cap vial (Kimble) and the methanol was removed by evaporation on the SpeedVac.

For derivatization, a hexane solution of trimethylamine was prepared by mixing 120 mg of trimethylamine hydrochloride (Sigma), 8.8 ml of purified H_2O, and 1.2 ml of 1 N NaOH in a 20-ml vial. Ten ml of hexane were added, the mixture was shaken, and the hexane was removed and dried with approximately 2 g of anhydrous Na_2SO_4. One ml of CH_2Cl_2 and 1 ml of hexane containing trimethylamine were added to the 4 ml vial containing the sample to be derivatized. The vial was shaken and 1 μl of pentafluorobenzoyl chloride (Aldrich) was added with a 1-μl disposable capillary pipet. It was shaken gently and then allowed to stand for 2 h at room temperature. The mixture was concentrated to dryness on the SpeedVac concentrator. This sample can be stored at −20°C for at least 1 week without significant decomposition.

The derivatized sample was dissolved in 70 μl of 1:1 THF:methanol containing 1 μg each of pentanophenone and hexanophenone (Pierce Chemical Co., Rockford, IL) as retention time markers. A 4.6-mm id x 12.5-cm Whatman Partisil 5 ODS-3 cartridge column with a Whatman guard column was used for isolation of the fraction containing HPB-*pentafluorobenzoate*. The solvent program was 35% methanol in H_2O for 10 min and then a linear gradient to 70% methanol in the next

15 min at 1 ml/min. The material eluting from approximately 28–31 min, corresponding to the apexes of the pentanophenone and hexanophenone peaks, was collected in a silanized 4-ml vial. The solvent was removed on the SpeedVac at approximately 36°C. The residue was taken up with 3 aliquots of 50 μl of THF and transferred to a silanized 250-μl conical vial (Kimble). The THF was removed on the SpeedVac and the sample was stored at −20°C until GC-NICI-MS analysis.

For GC-NICI-MS analysis, a 0.25-mm i.d. x 30-m 50% methylphenyl silicone (DB-17) bonded phase column (0.15 μm film thickness), connected to a 2-m, 0.32-mm i.d. fused silica uncoated deactivated retention gap was used. The column was connected to the retention gap with a Press-tight connector (Restek Corp., Bellafonte, PA). Injections were carried out with a septumless on-column injector (J and W Scientific, Folsom, CA). Injections were made with a 10-μl syringe equipped with a 5-inch fused silica needle. The needle was placed into the retention gap such that 2 inches extended into the GC oven. The sample was deposited at an injection speed of 1 μl/s onto the retention gap in an oven equilibrated at 35°C (31). The injection port temperature was 35°C and, after injection, the oven was programmed as follows: 35°C for 2.5 min; then 20°C/min to 150°C; then 4°C/min to 205°C; then hold for 20 min. The flow rate was 1 ml/min helium measured at 35°C.

Initially, we used either a splitless injection system or an on-column injection system equipped with stainless steel needles and silicone septa. However, shavings of the septa accumulated with time and interfered with the chromatography. Therefore, we adopted the septumless injection system. The use of a retention gap allowed us to inject relatively large sample volumes. The retention gap was periodically replaced to prevent accumulation of sample-related nonvolatile material.

Ten μl of ether containing 60 fmol of the external standard, HPB-tetrafluorobenzoate, were added to the 300-μl conical vial containing the derivatized sample. Then, the walls of the vial were washed with approximately 3 μl of ether. The volume of the ether solution was then measured by taking it up in a 10-μl syringe. The solution was placed back into the vial, and 50% of it was taken up with a fused silica needle syringe containing 1 μl of ether. It was injected on the column and the temperature program was initiated. The mass spectrometer was operated in the NICI mode with a methane pressure of 1.2 torr, ionizing energy of 100 eV, and a source temperature of 150°C. The molecular ions of HPB-pentafluorobenzoate (*m/e* 358.9), [4,4-D_2]HPB-pentafluorobenzoate (*m/e* 360.9), and HPB-tetrafluorobenzoate (*m/e* 340.9) were monitored.

Amounts of HPB in samples were calculated from the ratio of the integrated peak areas of the molecular ions of HPB-pentafluorobenzoate and [4,4-D_2]HPB-pentafluorobenzoate. In cases where the peak areas of these two molecular ions differed by more than a factor of 7, correction was made for the M+2 peak of HPB-pentafluorobenzoate, or the M-2 peak of [4,4-D_2]HPB-pentafluorobenzoate.

HPB in Hemoglobin of Rats Treated with Nicotine or [5-^{3}H]NNK. One male F344 rat (270 g) was treated by gavage with [5-^{3}H]NNK (1 μmol/kg, 0.2 mCi/μmol) in 1 ml of saline. A second rat (270 g) was given a gavage of nicotine (50 μmol/kg) in 1 ml of saline. They were killed 24 h later and blood was collected as described (23). The hemoglobin from the rat treated with [5-^{3}H]NNK was base treated and analyzed for [5-^{3}H]HPB by HPLC (23). The hemoglobin from the rat treated with nicotine was analyzed using the mass spectrometric method.

RESULTS

Fig. 2 outlines the analytical method. Mild base treatment of the dialyzed hemoglobin solution releases HPB. The separation of HPB from the protein greatly simplifies the analysis. After addition of [4,4-D_2]HPB as internal standard, a series of partitions is carried out to enrich the weakly basic fraction containing HPB. This fraction is derivatized with pentafluorobenzoyl chloride, which converts HPB to HPB-pentafluorobenzoate. The five fluorine atoms in this molecule make it amenable to detection by NICI-MS. The derivative is purified by HPLC and

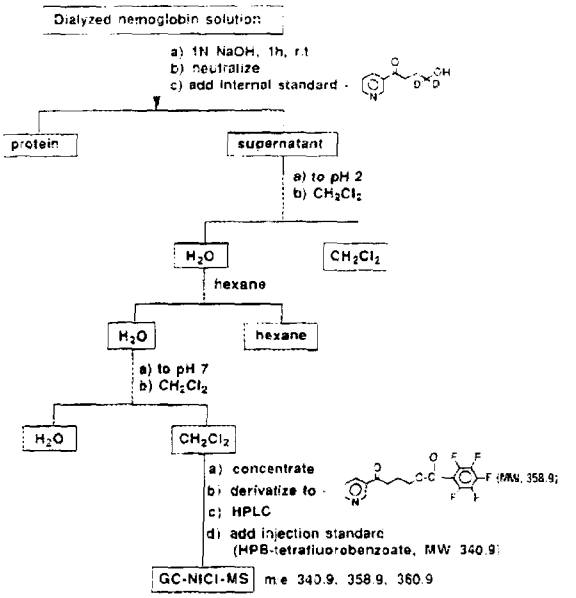

Fig. 2. Scheme for analysis of HPB in hydrolysates of human hemoglobin. *MW*, molecular weight.

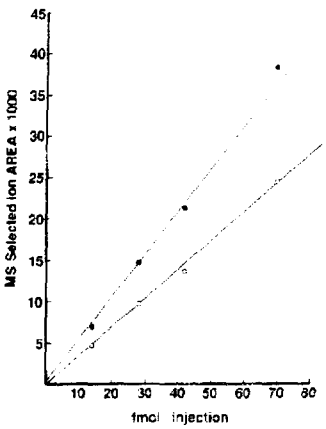

Fig. 3. GC-MS calibration curves for *m/e* 358.9 of HPB-pentafluorobenzoate (●) and *m/e* 360.9 of [4,4-D_2]HPB-pentafluorobenzoate (○).

the resulting fraction is analyzed by capillary column GC-NICI-MS. An external standard, HPB-tetrafluorobenzoate, is added at this stage to monitor GC-NICI-MS sensitivity.

Fig. 3 illustrates typical GC-NICI-MS standard curves for HPB-pentafluorobenzoate and [4,4-D_2]HPB-pentafluorobenzoate, monitored at their molecular ions, *m/e* 358.9 and 360.9. The response of the mass spectrometer was linear in the range used for sample analysis. The detection limit for HPB-pentafluorobenzoate was approximately 100 amol/injection or 5 fmol/g hemoglobin.

Fig. 4 illustrates representative GC-NICI-MS traces from analysis of hemoglobin obtained from a smoker and a snuff dipper. The HPB-pentafluorobenzoate and [4,4-D_2]HPB-pentafluorobenzoate peaks, marked with asterisks, were readily detected in all samples. In each sample, the appropriate peak eluted at the correct retention time as established by injection of standards. Samples were also analyzed on a 30-m DB-5 column (5% phenylmethyl silicone) and the retention time of the HPB-pentafluorobenzoate peak was identical to that of a standard. During the development of the method, several other chromatographic conditions were used and the HPB-pentafluo-

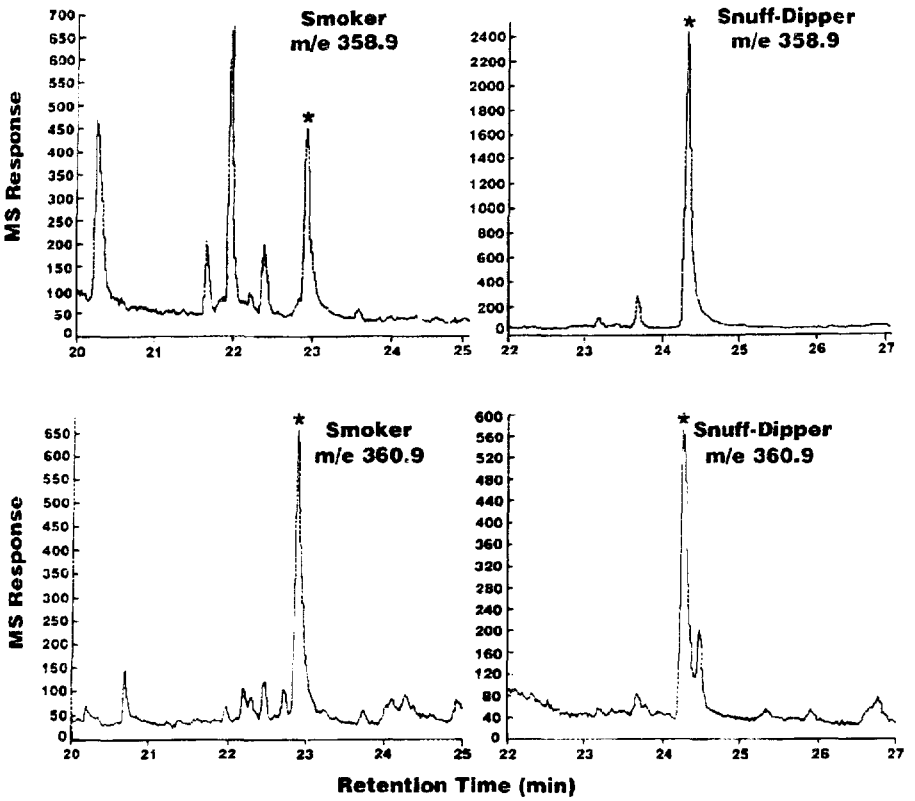

Fig. 4. Representative GC-NICI-MS traces of the HPB-pentafluorobenzoate fraction isolated from hemoglobin of (*left*) a smoker and (*right*) a snuff dipper. The traces were obtained by selected ion monitoring at *m/e* 358.9 and *m/e* 360.9, the molecular ions of HPB-pentafluorobenzoate and [4,4-D_2]HPB-pentafluorobenzoate. The peaks marked with the *asterisks* elute at the appropriate retention times. The retention times in the smoker and snuff dipper samples are different because slightly different conditions were used.

robenzoate peak had the correct retention time. The identities of the other peaks, which were frequently observed, are not known.

Most batches of eight samples from snuff dippers, smokers, or nonsmokers were analyzed at the same time as a positive control sample, prepared by adding 1570 fmol of HPB to 1 ml of hemoglobin solution. Analyses of these positive control samples gave 1490 ± 420 fmol (SD) HPB ($n = 10$). A composite snuff dipper sample was prepared by combining portions of individual samples such that the concentration of HPB was 1100 fmol/g hemoglobin. This sample was divided into five aliquots and each was analyzed; the mean value obtained was 1020 ± 18 fmol/g hemoglobin. A second snuff dipper sample was split into three aliquots and analyzed, giving a mean value of 1480 ± 63 fmol HPB/g hemoglobin. The recovery of [4,4-D_2]HPB was 26 ± 14% ($n = 104$).

Each batch of samples was also run in the presence of one or two H_2O blanks, to monitor possible contamination. Small amounts of HPB, 60.7 ± 37.4 fmol ($n = 11$), were found in these blank samples and were subtracted from the values obtained for each hemoglobin sample in that batch. The source of this background is not known.

Table 1 summarizes data obtained by analyses of HPB released from hemoglobin of snuff dippers. HPB levels ranged from not detected to 1830 fmol/g hemoglobin, with a mean of 517 ± 538 fmol/g hemoglobin ($n = 22$). There was no correlation between HPB levels and extent or duration of snuff usage or levels of salivary or plasma cotinine. Table 2 summarizes data from analyses of HPB released from hemoglobin of smokers and nonsmokers. Among the smokers, HPB levels ranged from not detected to 1160 fmol/g hemoglobin, with a mean of 79.6 ± 189 fmol/g hemoglobin ($n = 40$). There was no correlation of HPB levels with numbers of cigarettes smoked, levels of plasma cotinine, or age. The individuals with the seven highest HPB levels were all females. They smoked 12.8 ± 12.8 cigarettes/day and had plasma cotinine of 133 ± 97 ng/ml. Their mean HPB level was 336 ± 368 fmol/g hemoglobin. Among nonsmokers, HPB levels ranged from not detected to 95 fmol/g hemoglobin, with a mean of 29.3 ± 25.9 fmol/g hemoglobin ($n = 21$). The data for snuff dippers, smokers, and nonsmokers are compared in Fig. 5. HPB levels were significantly higher in snuff dippers than in smokers ($P < 0.0001$).

In order to determine whether nicotine might give rise to significant amounts of HPB bound to globin, rats were treated with 50 μmol/kg nicotine or 1 μmol/kg NNK. Hemoglobin was isolated and analyzed for HPB. It was not detected in the hemoglobin of the rat treated with nicotine. In the NNK-treated rat, the amount of HPB was 27 fmol/mg globin.

DISCUSSION

GC-NICI-MS has proved to be the most widely applicable method for detection and quantitation of hemoglobin adducts in humans. The most sensitive methods are those in which the analyte is removed from the hemoglobin molecule prior to analysis; this separates it from unmodified amino acids which are present in great excess. Methods of this type have been used to quantify methylated and hydroxyalkylated valine as well as aromatic amines bound to cysteine (24–26, 32, 33). The method reported here uses a similar strategy, allowing for high selectivity, sensitivity, and reproducibility. Since the measurements being made are in the parts per trillion range, *e.g.*, 1–300 pg HPB/g hemoglobin, and the actual amounts of HPB analyzed are typically 0.5–150 pg/sample, special precautions are necessary to avoid loss of analyte and to guard against sample contamination. Analyses carried out without the precautions described in "Materials and Methods" were unsatisfactory due to low recoveries or contamination.

Table 1 *HPB and cotinine in snuff dippers*[a]

Subject	Age (yr)	Snuff use			Cotinine (ng/ml)		HPB (fmol/g hemoglobin)
		Times/day	Min/dip	Yr of use	Salivary	Plasma	
1	21	4	20	3	45	ND[b]	907
2	21	2	60	4	223	ND	32
3	18	10	20	1	165	36	1830
4	18	10	30	4	185	42	434
5	19	3	20	2	315	17	1210
6	22	3	30	7	402	18	101
7	19	4	30	8	328	200	227
8	20	2	30	2	74	182	120
9	19	10	30	7	137	95	1040
10	19	2	60	10	158	81	182
11	18	5	40	5	125	88	1200
12	21	6	30	7	151	90	1480
13	19	4	20	1	210	ND	283
14	19	6	30	8	43	ND	671
15	22	1	20	4	10	ND	81
16	22	10	40	10	440	39	294
17	22	5	25	5	152	45	19
18	20	5	20	4	303	154	301
19	18	1	45	1	23	167	ND
20	19	3	45	1	118	17	741
21	21	5	30	5	224	17	230
22	18	4	20	3	ND	15	ND
Mean ± SD	19.8 ± 1.5[c]	4.7 ± 2.9	31.6 ± 12.2	4.6 ± 2.9	174 ± 124[c]	59.2 ± 64.4[d]	517 ± 538

[a] All dippers were male subjects at an eastern military academy. All used the same brand of snuff, except subjects 8, 14, and 19. Except for subjects 16 and 17, none reported any other tobacco use.
[b] ND, not detected.
[c] 0.99 nmol/ml.
[d] 0.34 nmol/ml.

The structure of the adduct which releases HPB upon base treatment is not known. As discussed in Ref. 2, it is probably not a cysteine adduct. Ongoing experiments indicate that the adduct is bound mainly to a globin β-chain and that it is formed *in vivo* from NNK and related model compounds not requiring metabolic activation, but not from HPB itself (34). These results support the mechanistic interpretation illustrated in Fig. 1. A concern was that nicotine metabolism might give rise to the same adduct. Although no known nicotine metabolism pathway could produce such an adduct (35), it was possible that a previously undiscovered minor pathway might exist. Since nicotine concentrations in tobacco and tobacco smoke exceed those of NNK and NNN by 3–4 orders of magnitude, a minor nicotine metabolism pathway could contribute to the levels of HPB released from smokers' or snuff dippers' globin. However, no evidence was found for HPB release from the globin of a rat treated with nicotine. Furthermore, the lack of correlation of cotinine levels with HPB levels in smokers and snuff dippers also supports our hypothesis that the adduct detected in humans is formed by metabolism of NNK and NNN, but not by metabolism of nicotine.

A remarkable and somewhat unexpected finding of this study was the relatively high adduct levels in snuff dippers compared to smokers. The snuff used by most of the participants in this study typically contains 0.7 and 9 μg/g dry weight of NNK and NNN, respectively (36). Average use was 4.7 times/day. Assuming that each dip contained 0.5 g dry weight of snuff (37), exposure to NNK would be approximately 1.6 μg/day, and NNN 21.1 μg/day. In rats treated with NNN, the release of HPB from globin is only about 16% as great as from NNK (23). Therefore, the effective daily dose producing HPB from NNK and NNN can be estimated as 1.6 μg + 0.16 × 21.1 μg, or 5 μg/day (0.07 μg/kg/day). Mainstream cigarette smoke typically contains 200 ng/cigarette each of NNK and NNN (38). The smokers in this study used an average of 21.8 cigarettes/day. Applying the same calculation, the effective dose producing HPB would be approximately 5 μg/day (0.07 μg/kg/day), similar to that from snuff dipping. On the basis of only these assumptions, one might have expected the mean adduct levels in snuff dippers and smokers to be similar. Instead, the mean adduct level was approximately 7 times higher in snuff dippers than in smokers.

Several hypotheses can be suggested to explain the higher levels of HPB released from the hemoglobin of snuff dippers compared to smokers. (*a*) Endogenous formation of tobacco-specific nitrosamines may occur more readily in snuff dippers than in smokers. Snuff contains nicotine, nornicotine, and nitrite which may be swallowed during dipping. Nitrosation, particularly of nornicotine, could readily occur in the acidic environment of the stomach (14, 16). This would lead to higher exposure to NNN than expected based only on analysis of snuff. (*b*) Binding to hemoglobin may occur more readily following p.o. administration of tobacco-specific nitrosamines, as in snuff dipping, compared to inhalation. No extensive comparative studies of adduct levels *versus* route of administration have yet been carried out in laboratory animals. In Ref. 1 as well as in our previous study (23), NNK and NNN were given by i.p. injection. (*c*) Adduct levels may be influenced by induction of drug-metabolizing enzymes by components of tobacco smoke which are not present in unburned tobacco. Thus, it is possible that chronic smoking, but not snuff dipping, induces enzymes which detoxify tobacco-specific nitrosamines. This could lead to higher adduct levels in snuff dippers than in smokers.

Regardless of the mechanism, these results provide supporting evidence for our hypothesis that tobacco-specific nitrosamines are causative agents for oral cavity cancer in snuff dippers (39). NNK and NNN are the only constituents of snuff known to induce oral tumors in rats (7). They have been detected in snuff dippers' saliva and are metabolized by cultured buccal mucosa (8, 10, 20). The results of this study indicate that snuff dippers can metabolically activate NNK and NNN to intermediates that bind to hemoglobin. Paper 2 (1) shows that formation of globin adducts and DNA adducts by NNK are correlated and on this basis it can be predicted that DNA adducts of tobacco-specific nitrosamines will be formed in exposed tissues of snuff dippers. It will be important to analyze these tissues

Table 2 *HPB and cotinine in smokers and nonsmokers*[a]

Smokers						Nonsmokers			
Subject	Sex	Age (yr)	Cigarettes/day	Plasma cotinine (ng/ml)	HPB (fmol/g hemoglobin)	Subject	Sex	Age (yr)	HPB (fmol/g hemoglobin)
1	M	22	20	248	ND[b]	1	F	41	35
2	M	64	20	247	62	2	F	31	42
3	F	39	40	364	13	3	F	47	25
4	F	45	3	ND	1160	4	F	58	ND
5	M	40	20	249	47	5	M	29	95
6	M	38	30	90	34	6	F	52	37
7	M	37	30	124	75	7	F	33	67
8	F	58	40	270	277	8	M	27	63
9	M	39	60	273	36	9	F	44	ND
10	F	54	30	120	ND	10	F	33	ND
11	F	56	30	258	ND	11	F	41	26
12	M	40	30	108	ND	12	F	54	13
13	M	48	20	390	19	13	F	24	14
14	M	37	30	235	44	14	F	29	21
15	M	29	15	104	107	15	F	50	49
16	M	38	20	279	30	16	F	36	ND
17	F	30	15	45	28	17	M	53	54
18	F	42	8	221	145	18	F	22	8.5
19	F	47	20	169	47	19	M	28	42
20	F	52	30	399	ND	20	M	29	13
21	F	37	20	187	ND	21	F	35	10
22	F	36	20	235	ND				
23	M	47	20	248	11	Mean ± SD		37.9 ± 10.9	29.3 ± 25.9
24	F	38	10	154	121				
25	F	56	15	177	37				
26	F	69	20	171	4.8				
27	F	43	20	313	ND				
28	F	53	20	208	ND				
29	F	40	7	196	13				
30	M	25	8	185	24				
31	F	32	16	34	245				
32	F	34	10	93	229				
33	F	45	3	159	175				
34	F	29	15	179	26				
35	M	55	40	253	ND				
36	F	43	20	240	13				
37	M	41	20	249	ND				
38	F	51	30	171	15				
39	F	49	15	29	76				
40	F	30	30	381	71				
Mean ± SD		42.7 ± 10.5	21.8 ± 11.3	201 ± 97[c]	79.6 ± 189				

[a] Nonsmoking status was confirmed by lack of plasma cotinine.
[b] ND, not detected.
[c] 1.1 nmol/ml.

for DNA adducts and determine their relationship to tumor development.

Five of 22 snuff dippers (23%) and 7 of 40 smokers (18%) had HPB levels which were clearly elevated compared to the other individuals in their respective groups. Previous studies have demonstrated that there are large interindividual differences in the metabolism of tobacco-specific nitrosamines by cultured human tissues (20). Our results indicate that some individuals can metabolically activate NNK and NNN more extensively than others. The factors influencing NNK and NNN metabolism in humans require further investigation. For example, all smokers with elevated HPB levels were females and their extents of smoking and plasma cotinine values were lower than those of the remaining smokers. At present, it is not clear whether this is a general phenomenon or a characteristic of this particular sample of smokers.

Table 3 summarizes representative data from studies of hemoglobin adducts of aromatic amines, ethylene oxide, and methylating agents in smokers and nonsmokers. HPB levels in nonsmokers are lower than those of other hemoglobin adducts because HPB is formed from tobacco-specific compounds. Some of the HPB detected in samples from nonsmokers is probably due to sample contamination, although the amounts of HPB detected in H_2O blanks have been subtracted. We are presently investigating the origins of the HPB background. Elimination of this background will make the method even more sensitive and may result in a clearer distinction between smokers and nonsmokers.

HPB levels in smokers are also lower than those of other hemoglobin adducts reported to date. For compounds such as 4-aminobiphenyl and ethylene oxide, the observed levels reflect their concentrations in cigarette smoke as well as their extents of binding to hemoglobin. For example, measured amounts of 4-aminobiphenyl in cigarette smoke are about 5 ng/cigarette, about one-fortieth of NNK levels (40); however, the binding of this compound to cysteine of hemoglobin amounts to 5% of the dose in rats (41), compared to 0.02% of the dose released as HPB in rats treated with NNK (23). Therefore, one might expect that 4-aminobiphenyl adducts would be 5–10 times higher than tobacco-specific nitrosamine adducts in smokers. The data in Table 3 are consistent with this expectation. Ethylene concentrations in cigarette smoke are approximately 1000 times as great as those of NNK (24). For several of the other adducts in Table 3, such as those formed from aniline and methylating agents, sources other than tobacco smoke make major contributions to the observed levels (32).

In rats, NNK methylates and pyridyloxobutylates DNA (42, 43). The relationships of these processes to dose and to hemo-

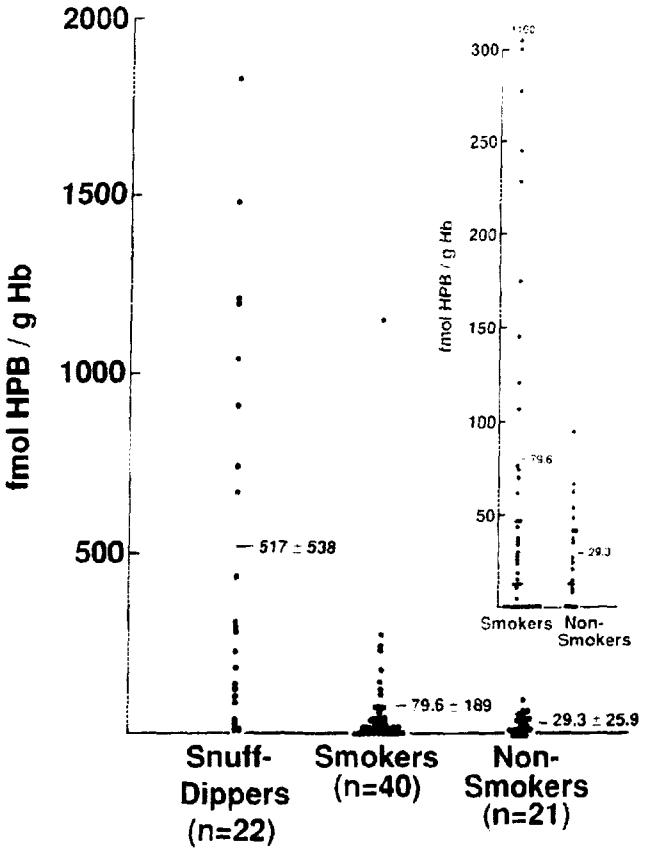

Fig. 5. Levels of HPB released from hemoglobin (*Hb*) of snuff dippers, smokers, and nonsmokers.

Table 3 *Representative hemoglobin adduct levels in smokers and nonsmokers*

Compound analyzed	Adduct levels (fmol/g hemoglobin) Smokers	Nonsmokers	Ref.
HPB	79.6 ± 189	29.3 ± 25.9	
4-Aminobiphenyl	911 ± 278	166 ± 77	26
2-Aminonaphthalene	100 ± 50	40 ± 20	33
o-Toluidine	930 ± 280	320 ± 90	33
m-Toluidine	4,600 ± 1,600	6,400 ± 1,900	33
p-Toluidine	1,200 ± 470	640 ± 370	33
Aniline	47,000 ± 25,000	41,000 ± 22,000	33
N-(2-Hydroxyethyl)valine	389,000 ± 138,000	58,000 ± 25,000	24
N-Methylvaline	540,000 ± 90,000	500,000 ± 10,000	32
N'-Methylhistidine	9,200,000 ± 6,300,000	25,000,000 ± 14,000,000	32

globin alkylation are described in Paper 2 (1). O^6-Methylguanine has been implicated as one important DNA adduct in NNK-induced lung carcinogenesis (44). The role of DNA pyridyloxobutylation in NNK carcinogenesis is not well understood. Therefore, it would be important to be able to estimate O^6-methylguanine levels in potentially susceptible cells or tissues of tobacco users. Since DNA is not readily available in adequate quantities for such assays, one approach would be measurement of hemoglobin methylation as a surrogate. However, previous studies indicate that this is impractical with presently available methods because of the high levels of endogenous methylation as well as methylation from sources other than tobacco, as illustrated in Table 3 (32). In contrast, measurement of HPB levels provides a tobacco-specific dosimeter that may be related in a predictable way to both DNA pyridyloxobutylation and methylation by NNK.

In summary, this study has provided the first measurements of tobacco-specific nitrosamine hemoglobin adducts in humans. Adduct levels were highest in snuff dippers, followed by smokers and nonsmokers. In snuff dippers and in a subgroup of smokers, adduct levels were substantially higher than would have been predicted based only on estimates of dose. The results of this study provide new leads for understanding the metabolic activation of tobacco-specific nitrosamines in humans.

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