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Optimized Liquid Chromatography Nanoelectrospray-High Resolution Tandem Mass Spectrometry Method for the Analysis of 4-Hydroxy-1-(3-pyridyl)-1-butanone-Releasing DNA Adducts in Human Oral Cells

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Abstract

Metabolic activation of the carcinogenic tobacco-specific *N*-nitrosamines leads to the formation of 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB)-releasing DNA adducts. We recently developed a liquid chromatography (LC)-tandem mass spectrometry (MS/MS) method for the analysis of HPB-releasing DNA adducts in human oral cells. However, given the limited amounts of DNA that can be extracted from oral cells, higher sensitivity and selectivity are required for the reliable analysis of these adducts in future studies. We have developed a new sensitive LC-nanoelectrospray ionization-high resolution MS/MS method for the analysis of HPB-releasing DNA adducts in oral cells. A new procedure was also developed for guanine analysis by LC-MS/MS. The detection limit of the developed assay is 5 amol, and the limit of quantitation is 0.35 fmol HPB on-column, starting with 50 pg DNA. The method was tested by analyzing oral samples from 65 smokers, including 30 head and neck squamous cell carcinoma (HNSCC) patients and 35 cancer-free controls. In all smokers, the levels of HPB-releasing DNA adducts averaged 6.22 ± 16.18 pmol/mg DNA, with significant inter-individual variation being consistent with previous reports. The median HPB-releasing DNA adduct level was 6.6 time greater for those with HNSCC than for smokers without HNSCC ($p=0.002$). The developed highly sensitive and selective method is valuable tool for future measurement of HPB-releasing DNA adducts in tobacco users, which can potentially provide critical insights for the identification of individuals at risk for cancer.

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Notes

The authors declare no competing financial interest.

Supporting Information

Product ion spectra of HPB and [pyridine-D₄]HPB; Typical SRM chromatograms of guanine; Linearity of guanine. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

HPB; DNA adduct; oral cells; oral cancer; nanoelectrospray; high resolution mass spectrometry

INTRODUCTION

Exposure to chemical carcinogens present in tobacco and cigarette smoke is largely responsible for the high cancer morbidity and mortality rates among tobacco users worldwide.¹⁻³ DNA adduct formation is central to the process of chemical carcinogenesis.^{4,5} In exposed individuals, the levels of DNA adducts derived from a specific carcinogen are likely to depend on the level of exposure, the relative efficiency of the carcinogen metabolic activation and detoxification pathways, as well as the efficiency of DNA repair mechanisms.³ Therefore, measurement of tobacco carcinogen-derived DNA adducts can potentially provide critical insights into individual susceptibility to tobacco-induced cancers in tobacco users.

Tobacco-specific *N*-nitrosamines (TSNA) are among the most important carcinogens in tobacco and cigarette smoke. Two compounds from this group – 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*'-nitrosonornicotine (NNN) – are organ-specific carcinogens inducing cancers of the lung, pancreas, oral and nasal cavity, and esophagus, and are classified as carcinogenic to humans by the International Agency for Research on Cancer.^{6,7} Since NNK and NNN are formed from tobacco alkaloids, human uptake of these carcinogens occurs primarily through exposure to tobacco or cigarette smoke, and to a lesser degree, from some tobacco alkaloid-containing products such as nicotine replacement therapy products or electronic cigarettes.^{6,8-10} Measurement of urinary biomarkers of NNK and NNN exposure generated critical insights into human uptake and metabolism of these carcinogens.^{8,11,12} Notably, recent studies demonstrated the association between NNK and NNN exposure and the risk of lung and esophageal cancer, respectively, in smokers from the prospective Shanghai cohort study.^{11,13}

Metabolic activation of NNK and NNN leads to the formation of pyridyloxobutyl (POB) DNA adducts.^{4,6} Under strong acid hydrolysis conditions, the majority of POB-DNA adducts decompose to release 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB).¹⁴⁻¹⁶ In vitro and laboratory animal studies have demonstrated the importance of HPB-releasing adducts in NNK- and NNN-induced carcinogenesis.^{6,17} A limited number of studies measured HPB-releasing DNA adducts in human tissues, supporting the potential utility of this biomarker as a direct measure of tobacco-induced DNA damage and cancer risk.¹⁸⁻²¹ The recently proposed use of oral cells as a surrogate source of DNA for HPB-releasing DNA adduct measurement in smokers presents a promising approach to overcome the invasiveness of tissue collection from the most organs targeted by NNK and NNN.¹⁶ In addition, given that NNN is a potent oral carcinogen,¹⁷ measurement of HPB-releasing DNA adducts in oral cells could potentially provide the most direct quantitative and mechanistic link between tobacco use and oral cancer in humans.

We recently developed a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the analysis of HPB-releasing DNA adducts in human oral cells.¹⁶ However,

application of this method in studies in which only limited oral cells are available for DNA extraction requires further modification to increase its sensitivity and selectivity. Collection of oral brushings from oral cancer patients or from children exposed to secondhand smoke are some examples of scenarios in which only small amounts of DNA may be extracted from the collected samples. Our purpose in this study was to modify the previously developed method by optimizing the sample preparation procedure and switching to LC-nano electrospray ionization-high resolution tandem mass spectrometry (LC-NSI-HRMS/MS). To demonstrate the utility of this optimized method, we applied it to the analysis of oral brushings collected from smokers with head and neck squamous cell carcinoma (HNSCC). In addition, since in our previous study smokers with HNSCC showed increased exposures to NNN than those without HNSCC,²² oral samples from cancer-free smokers were analyzed here for comparison. The ultimate goal of the development of this methodology is its application in future studies to understand the relationship between tobacco exposure and HPB-releasing DNA adduct formation in tobacco users, and to evaluate the utility of these adducts as biomarkers of cancer risk in tobacco users.

EXPERIMENTAL PROCEDURES

Chemicals and enzymes

HPB, [pyridine-D₄]HPB, [3,3,4,4-D]HPB, and [¹³C₂¹⁵N]guanine were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Reagents and enzymes for DNA isolation were obtained from QIAGEN Sciences (Germantown, MD). Calf thymus DNA was purchased from Worthington Biochemical Corporation (Lakewood, NJ). All other chemicals and solvents were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI).

Subjects and oral cell collection

Oral brushings were collected from 30 smokers with head and neck squamous cell carcinoma (HNSCC) and 35 smokers without HNSCC recruited through the Department of Otolaryngology-Head and Neck Surgery at the University of Minnesota. Study participants were included if they were daily smokers of at least 5 cigarettes per day for at least 5 years. HNSCC patients were identified upon presentation to the Otolaryngology-Head and Neck Surgery clinic with a new diagnosis of squamous cell carcinoma of the upper aerodigestive tract. This included tumors of the oral cavity, oropharynx, larynx and hypopharynx. Cancer-free controls were recruited in the same outpatient clinic. These subjects were visiting the clinic for clinical evaluation of problems other than cancer (i.e. sinusitis, hearing loss). The study was approved by the University of Minnesota Research Subjects' Protection Programs Institutional Review Board: Human Subjects Committee (IRB Study # 0903M62203). Oral cells were collected by brushing the oral mucosa inside one cheek with a clean toothbrush and swirling the brush in a sterile polypropylene centrifuge tube with a commercial mouthwash to transfer the collected buccal cells from the brush into the liquid. After the collection, the samples were centrifuged at 2,700 rpm to pellet cells; the pellets were washed with Tris-EDTA buffer (pH 7.4) and stored at -20 °C until DNA isolation and analysis.

DNA isolation from oral cells

DNA isolation from the collected samples was performed using the commercial protocol for DNA purification from oral cells (Qiagen, Valencia, CA). Briefly, oral cells were thawed and centrifuged at $3,000 \times g$ for 15 min at 4°C . Supernatant was discarded and pellets were re-suspended in 200 μL 100 mM phosphate buffer (pH 6.8). Cells were lysed using Qiagen AL buffer, treated with proteinase K and RNase A, and DNA was purified using QiaAmp DNA Mini spin columns. The amount of isolated DNA was calculated based on the amount of guanine,²³ which was analyzed as part of our developed method as described below.

DNA hydrolysis and sample purification

The isolated DNA samples were mixed with 0.12 pmol of [pyridine- D_4]HPB and 1000 pg of [$^{13}\text{C}_2^{15}\text{N}$]guanine, which served as internal standards for HPB and guanine, respectively. A water blank (0.5 mL) and a sample of calf thymus DNA (5 μg dissolved in 0.5 mL deionized water) were added to each sample set as negative controls. Hydrochloric acid was added to each sample to bring the final concentration of the acid to 0.8 N, and the samples were incubated at 80°C for 3 h to release HPB.^{14, 16, 20} The hydrolysates were adjusted to neutral pH with NaOH, and loaded on HyperSep Hypercarb cartridges (25 mg, Thermo Scientific, Rockwood, TN) activated with 2 mL MeOH and 2 mL H_2O . The cartridges were then washed with 2 mL H_2O . HPB was eluted with 1 mL of 65% MeOH, and guanine was eluted with 1 mL MeOH (containing 1% TFA) sequentially. The two fractions were collected in separate collection tubes, concentrated to dryness in a centrifugal evaporator, and re-dissolved in H_2O prior to mass spectrometry analysis.

Analysis of released HPB by LC-NSI-HRMS/MS

The analysis of HPB in the prepared samples was carried out on an LTQ Orbitrap Velos instrument (Thermo Scientific, Waltham, MA) interfaced with a Nano2D-LC HPLC (Eksigent, Dublin, CA) system using nanoelectrospray ionization. The analysis was performed using a capillary column (75 μm ID, 10 cm length, 15 μm orifice) prepared by hand packing a commercially available fused-silica emitter (New Objective, Woburn MA) with Luna C18 bonded separation media (Phenomenex, Torrance, CA). The mobile phase consisted of 5 mM NH_4OAc and CH_3CN , using a linear gradient at a flow rate of 300 nL/min with increasing CH_3CN from 2 to 30% over 15 min, followed by ramping to 98% CH_3CN within 1 min, and holding at this composition for 2 min. The gradient was then returned to 2% CH_3CN in 1 min and the system was re-equilibrated at this mobile phase composition for 5 min at 1000 nL/min before next injection. For injections, a 5 μL injection loop was used and the sample (4 μL) was loaded onto the capillary column with a 1000 nL/min flow at the initial conditions (2% CH_3CN) for 5.5 min. The nanoelectrospray source voltage was set at 2.2 kV. The capillary temperature was 350°C , and the S-Lens RF Level was set at 40%. The analysis was performed using accurate mass extracted ion chromatograms of m/z 106.0287 [$\text{C}_6\text{H}_4\text{NO}$]⁺ (parent ion m/z 166.1) for HPB and corresponding fragment (m/z 110.0538, parent ion m/z 170.1) for [pyridine- D_4]HPB with a mass tolerance of 5 ppm. The scan events were performed using higher-energy collisional dissociation (HCD) fragmentation with a normalized collision energy of 60 units, isolation

widths of 3 Da for both the analyte and internal standard, and product ion spectra acquisition at a resolution of 50,000.

Analysis of guanine by LC-MS/MS

Analysis of guanine was performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, Waltham, MA) interfaced with an Agilent 1100 capillary HPLC system (Agilent, Palo Alto, CA). Analysis was performed on a Luna C18(2) column (5 μ m, 250 \times 0.5 mm, Phenomenex) at a flow rate of 10 μ L/min with the temperature maintained at 30°C. Sample injection volume was 8 μ L. The mobile phase consisted of H₂O (containing 0.01% acetic acid) and CH₃CN with a linear gradient from 3 to 18% CH₃CN over a period of 15 min, followed by ramping to 70% CH₃CN within 1 min and holding at this composition for 4 min. The gradient was then returned to 3% CH₃CN in 2 min followed by 15 min re-equilibration. The ESI source was operated in positive ion mode, monitoring m/z 152 [M + H]⁺ \rightarrow 135 [C₅H₃N₄O]⁺, and m/z 152 \rightarrow 110 [C₄H₄N₃O]⁺ for guanine, and corresponding transitions m/z 155 \rightarrow 138 and m/z 155 \rightarrow 113 for [¹³C₂¹⁵N]guanine. The collision gas was Ar at 1 mTorr with collision energy of 19 eV. The quadrupoles were operated at a resolution of 0.2 (Q1) and 0.7 (Q3) Da.

Quantitation of HPB and guanine

The quantitation of HPB and guanine was based on the peak area ratio of the analytes to their corresponding isotope-labeled internal standards, the constructed calibration curves, and the amount of internal standards added. Calibration curves were constructed before each analysis using a series of standard solutions of analytes and internal standards. The calibration standard solutions of HPB contained a constant amount of [pyridine-D₄]HPB (4.7 fmol on-column) and varying amounts of HPB (0.005, 0.012, 0.024, 0.05, and 0.12 fmol on-column). The calibration standard solutions of guanine contained a constant amount of [¹³C₂¹⁵N]guanine (80 ng on-column) and varying amounts of guanine (1.6, 4, 8, 40, 80, 400, and 800 ng on-column).

Method characterization

To eliminate any potential interference of background HPB contamination, we used [pyridine-D₄]HPB to characterize the accuracy and precision of the developed method. To determine the amounts of [pyridine-D₄]HPB in the samples processed in these experiments, we used [3,3,4,4-D₄]HPB as internal standard. The two deuterium-labeled standards have the same parent ion m/z 170.1, but form different fragments and therefore can be distinguished by extracting ion chromatograms of m/z 110.0538 for [pyridine-D₄]HPB and m/z 106.0287 for [3,3,4,4-D₄]HPB. Accuracy and precision were determined by adding different amounts of [pyridine-D₄]HPB (0.35, 0.7, 3.5, 7, and 35 fmol) and 3.5 fmol of [3,3,4,4-D₄]HPB to 50 μ g calf thymus DNA in 0.5 mL H₂O, followed by hydrolysis and purification as described above. Samples at each level of added [pyridine-D₄]HPB were prepared and analyzed in triplicate.²⁴ Accuracy was determined by comparing added and measured amounts of [pyridine-D₄]HPB at each level. Precision was determined as intra-day and inter-day coefficients of variation (% CV) for the triplicate samples analyzed on three separate days.

The limit of detection (LOD) was determined using standard solutions of [pyridine-D₄]HPB. The limit of quantitation (LOQ) was established in calf thymus DNA samples by adding [pyridine-D₄]HPB (0.07, 0.35, 0.7, and 3.5 fmol) and [3,3,4,4-D₄]HPB (3.5 fmol) to calf thymus DNA samples, followed by hydrolysis and purification, and analyzing each sample in triplicate. The LOQ was defined by identification of the lowest [pyridine-D₄]HPB level that produced a coefficient of variation (CV) lower than 15%.²⁵

Recovery was determined by comparing the results of samples to which [3,3,4,4-D₄]HPB (240 pmol) was added to 5 µg of calf thymus DNA at the beginning and at the end of sample preparation procedure.

Statistical analyses

The HPB values below the LOQ were estimated based on the lowest quantifiable HPB level among the analyzed oral samples (see footnote 'b' to Table 1). DNA yields and the HPB-releasing DNA adduct levels were summarized by the mean, standard deviation (SD), median and inter-quartile range (IQR) for HNSCC patients and cancer-free smokers separately and combined. Due to an extremely skewed distribution for the HPB level, the analysis was performed in the natural logarithmic scale using a two-sided, two-sample t-test with unequal variances. To verify these results, the two-sample non-parametric Wilcoxon rank sum test was applied to the original arithmetic values without imputing a value below the LOQ, but instead assigning the same lowest rank for these cases. The Fisher's exact test compared the percent of cases with HPB-releasing DNA adducts below the LOQ between cancer and cancer-free smokers.

RESULTS

Development of the analytical procedure

The developed protocol is outlined in Figure 1. Changes in sample purification procedure introduced in this modified method were driven in part by the need to optimize DNA quantitation in samples obtained from oral brushings. In our previously reported method, an aliquot (40 µL) of unpurified DNA hydrolysate from each sample was collected for analysis of guanine by HPLC-UV,¹⁶ which is used to calculate DNA yield.²³ However, due to the small amounts of DNA that can be obtained from oral cells collected with a cytobrush, we were not able to measure guanine reliably by HPLC-UV in most samples. The development of a more sensitive and selective mass spectrometry-based method required introduction of a solid phase extraction clean-up step for hydrolysate purification to remove interfering salts and thus prevent ion suppression during guanine analysis. Initially, we explored the use of Strata X (Phenomenex) – the cartridge used in our originally developed method – to wash out interfering salts in H₂O and subsequently elute both guanine and HPB by applying stronger solvents. However, guanine was not retained on the cartridge due to its high polarity. Therefore, we switched to Hypercarb solid phase extraction cartridge (Thermo) which has high affinity towards polar analytes. Both guanine and HPB present in DNA hydrolysates were successfully retained on the cartridge; the analytes were eluted from the cartridge in two wash-out steps (Figure 1), generating two separate fractions for mass spectrometry analyses.

Analysis of HPB released from DNA—Using HCD fragmentation of the LC-NSI-HRMS/MS system, the product ion spectra of HPB and [pyridine-D₄]HPB generated several major fragment ions (Figure S1). Because the highest signal intensities in the spectra of both HPB and [pyridine-D₄]HPB were for m/z 106.0287 and m/z 110.0538, respectively, these fragments were selected for quantitative analysis. Two additional fragments, m/z 148.0757 and m/z 122.0600, were used to confirm the identity of HPB; the corresponding deuterium-labeled fragment ions were used to confirm the identity of [pyridine-D₄]HPB.

Analysis of guanine—Based on the fragmentation patterns determined in the product ion scan mode, two major ions were monitored for guanine and its internal standard in selected reaction monitoring (SRM) mode: m/z 135 and m/z 110 for guanine, and m/z 138 and m/z 113 for [¹³C₂¹⁵N]guanine. Because of their higher signal intensities, the transitions m/z 152→135 and m/z 155→138 were selected for quantitation of guanine and [¹³C₂¹⁵N]guanine, respectively. The peak area ratio between m/z 152→135 and m/z 152→110 was used to confirm the identity of guanine, and corresponding ratio was used to confirm the identity of the internal standard (Figure S2). With this developed LC-ESI-MS/MS method, guanine exhibited excellent linearity within the determined concentration range (Figure S3), and it was accurately quantified in all oral cell DNA samples. Typical SRM chromatograms obtained upon analysis of guanine and [¹³C₂¹⁵N]guanine in a standard mix and in oral cells from a smoker are presented in Figure 3.

Method characteristics

The average accuracy of measured levels of [pyridine-D₄]HPB (expressed as % of added amount) at 0.35, 0.7, 3.5, 7, and 35 fmol was 106%, 116%, 108%, 107%, and 109%, respectively, exhibiting excellent linearity ($R^2 = 1$, Figure 4A). The interday and intraday CVs were 11% and 13%, respectively. The recovery of the assay was $65 \pm 4\%$ ($n=5$). An LOD of 5 amol [pyridine-D₄]HPB (on-column) was achieved for a standard HPB solution. In CT-DNA samples mixed with HPB, an LOQ of 0.35 fmol (on-column) was achieved based on a CV of 13%. During the analysis of oral samples from smokers, the instrument response and the HPB/[pyridine-D₄]HPB ratio for the calibration standard mixes were linear in the 0.005–0.12 fmol (on-column) range of HPB ($R^2 = 0.998$, Figure 4B).

Quantitation of HPB-releasing DNA adducts in human oral cells

The developed method was applied to the analysis of oral cell DNA samples from 65 smokers, including 30 smokers with HNSCC and 35 smokers without HNSCC. Typical chromatograms obtained upon analysis of HPB in oral cells from a smoker with HNSCC and a smoker without HNSCC are presented in Figure 5.

The results of HPB levels from all the subjects are listed in Table 1. The yield of DNA isolated from oral cells in this study averaged $0.53 \pm 1.16 \mu\text{g}$ (median 0.18, IQR 0.063, 0.49). The levels of HPB in the analyzed samples ranged from non-quantifiable to 92.2 pmol/mg DNA, averaging (\pm SD) 6.22 ± 16.18 pmol/mg DNA in all subjects (median 0.68, IQR 0.028, 2.56). Only one out of 30 (3.3%) HNSCC patients had HPB-releasing DNA adduct levels below the LOQ, compared to 15 out of 35 (42.9%) cancer-free smokers ($p<0.001$). In HNSCC patients, HPB-releasing DNA adducts averaged 8.19 ± 18.12

pmol/mg DNA (median 1.51, IQR 0.32, 5.12). In cancer-free smokers, the adduct level averaged 4.53 ± 14.36 pmol/mg DNA (median 0.23, IQR 0.001, 1.61). Comparing the means in the log scale resulted in a significantly higher HPB-releasing DNA adducts in HNSCC patients than cancer-free smokers ($p < 0.001$). Comparing the medians plus percentiles between these groups using the non-parametric method resulted in a similar finding, with the median HPB-releasing DNA adducts for the cancer cases ~6.6 times greater than non-cancer smokers ($p = 0.002$).

DISCUSSION

Analysis of HPB-releasing DNA adducts in oral cells can potentially be used as a non-invasive measure of DNA damage caused by the tobacco carcinogens NNK and NNN in tobacco users. We previously developed a method for the analysis of HPB-releasing DNA adducts in human oral cells. However, the amount of DNA that can be isolated from oral cells is usually low, and application of the previously developed method in our new studies often resulted in undetectable levels of HPB or inability to reliably quantify DNA in the available oral samples. Therefore, we aimed to modify our previously developed method in order to achieve lower limit of quantitation and better selectivity for HPB-releasing DNA adducts, as well as higher accuracy of DNA quantitation in human oral cells.

The previous method for quantitation of HPB in oral cells was based on the use of a triple quadrupole mass spectrometer and achieved an LOQ of 2.4 fmol HPB on-column.¹⁶ In that study, a high resolution mass spectrometer (HRMS) with accurate mass capability (Orbitrap) was used to confirm the identity of HPB in several oral cell DNA samples.¹⁶ That experiment revealed that when the same processed oral cell sample was analyzed on the two instruments, a dramatic improvement in selectivity was observed in the case of HRMS, producing clear peaks and no baseline noise or co-eluting interferences. Based on this observation, and our previous successful experience with the quantitation of another DNA adduct, 3-(2-deoxy- β -D-erythro-pentafuranosyl)pyrimido[1,2- α]purin-10(3H)-one deoxyguanosine (M₁dG),²⁶ we decided to switch to the use of LC-NSI-HRMS/MS for the development of the new optimized method. An LOQ of 0.35 fmol on-column was achieved using the current method, improving the sensitivity by approximately 7-fold compared to previous method. Consistent with the observation from our first study, the baseline noise was completely eliminated with the use of LC-NSI-HRMS/MS, greatly improving selectivity of the method (Figure 5). In addition to the improvement in the measurement of HPB-releasing DNA adducts, our procedure allows for simultaneous extraction of guanine during solid-phase purification of DNA hydrolysates, and the quantitation of guanine by the newly developed mass spectrometry-based method. Among the analyzed samples, the lowest calculated DNA yield based on guanine analysis was 0.002 μ g (Table 1), demonstrating excellent sensitivity of the developed method and its value for studies in which only limited amounts of DNA are available.

Application of the modified method to the analysis of oral cells from smokers showed large inter-individual variation in the levels of HPB-releasing DNA adducts, which encourages further research on the role of these adducts in cancer risk in tobacco users. This variation and the range of adduct levels – from non-quantifiable to 92.2 pmol/mg DNA – are

consistent with our previous report.¹⁶ In that report, one out of 30 oral cell brushings from smokers contained HPB in the amount of 246 pmol/mg DNA; the remaining 29 samples ranged from non-quantifiable to 109 pmol/mg DNA, which is similar to the current study. The average level of HPB-releasing DNA adducts in the current study however, is 6.22 ± 16.18 pmol/mg DNA in all 65 subjects, which is lower than 45 ± 57 pmol/mg DNA in 30 smokers in the previous report. This difference could be due to the differences in personal characteristics (for instance smoking level and history) across the two groups of smokers: the previous study used samples collected through the NCI-supported TobPRAC biorepository, while the subjects for the current study were recruited in the outpatient clinic at the University of Minnesota, and the time of subject recruitment and sample collection is at least 5 years apart for the two studies. Independent of the reason, the lower average levels of HPB-releasing DNA adducts, as well as the slightly lower DNA yields, in this study as compared to the previous report further stress the importance of our new highly sensitive methodology for future investigations.

In this study, we included smokers with and without HNSCC to provide preliminary insights into the potential differences in HPB-releasing DNA adduct levels between oral cancer patients and cancer-free smokers. The comparison between the two groups of smokers shows that HPB-releasing DNA adducts were detected in 97% of HNSCC patients and only 57% of cancer-free smokers, with the median levels being more than 6-fold higher in the oral cells of HNSCC patients than in controls (Table 1). This difference is highly statistically significant; however, these results should be validated with further investigations. Tobacco is a major etiologic factor in the development of oral cancer, with approximately 75% of all oral cancer patients being tobacco users,²⁷⁻²⁹ and NNN is a potent oral carcinogen. Furthermore, our previous study has shown that urinary total NNN was higher in smokers with HNSCC than in cancer-free smokers.²² In the present study, all enrolled subjects completed an in-depth questionnaire on lifetime tobacco use and provided additional biological samples, including urine. While in-depth analysis of smoking histories and constituent exposures is beyond the scope of this report, additional research is underway to compare these measures between the two groups of smokers and investigate the potential utility of oral cell HPB-releasing DNA adducts in identifying tobacco users at risk for oral cancer. Given the high inter-individual variability of HPB-releasing adduct levels with some being below the LOQ, future studies comparing different tobacco and disease groups could utilize the Tobin model,³⁰ which allows for the uncertainty about the unobserved values below the LOQ and permits the addition of multiple covariates related to subject characteristics to explain more of the inter-individual variations in the HPB-releasing DNA adducts.

In summary, we have developed an improved highly sensitive LC-NSI-HRMS/MS method for the analysis of HPB-releasing DNA adducts in small amounts of oral cell DNA, and successfully applied this method to the analysis of oral samples from 65 smokers. This methodology can be applied in future studies on the role of HPB-releasing DNA adduct formation in tobacco-induced cancers in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CV	coefficient of variation
ESI	electrospray ionization
HCD	higher-energy collisional dissociation
HNSCC	head and neck squamous cell carcinoma
HPB	4-hydroxy-1-(3-pyridyl)-1-butanone
IQR	inter-quartile range
LC-NSI-HRMS/MS	liquid chromatography-nanoelectrospray ionization-high resolution tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantitation
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N'-nitrosornicotine
POB DNA adducts	pyridyloxobutyl DNA adducts
SD	standard deviation
SRM	selected reaction monitoring
TSNA	tobacco-specific nitrosamines

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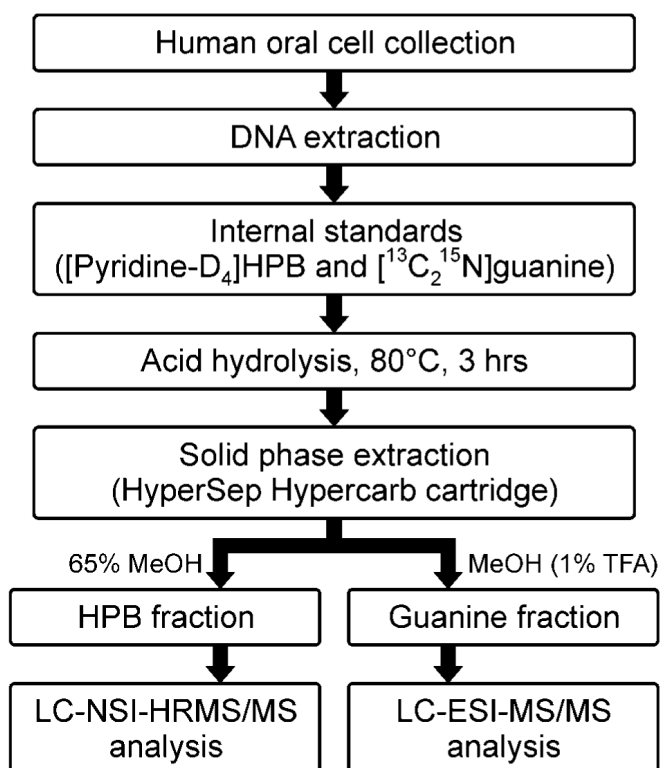


Figure 1. Analytical scheme for the analysis of HPB-releasing DNA adducts and guanine in human oral cell DNA.

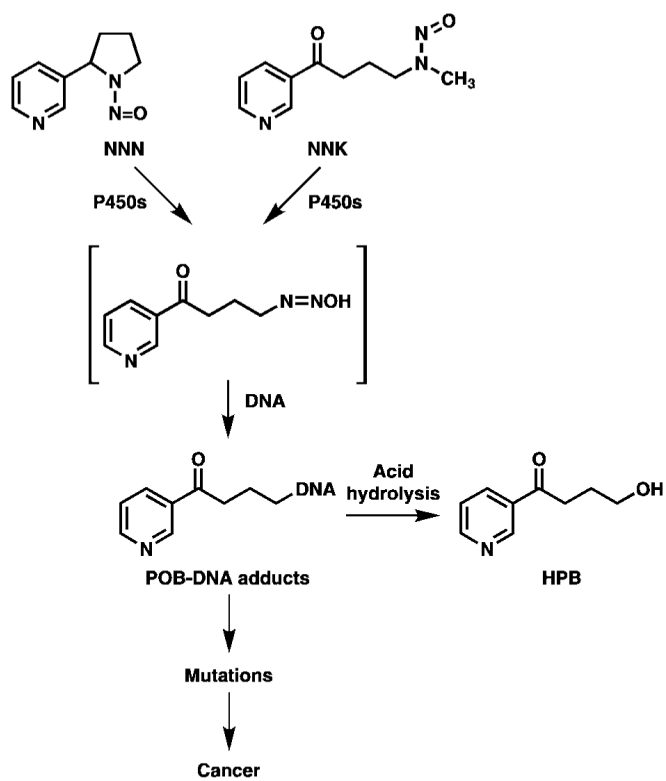


Figure 2.
Formation of HPB-releasing DNA adducts from NNK and NNN.

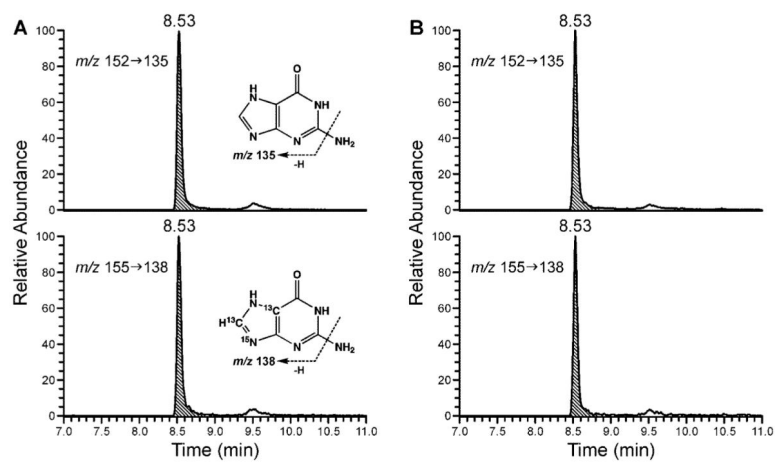


Figure 3. Typical SRM chromatograms obtained upon analysis of guanine and $[^{13}\text{C}_2^{15}\text{N}]$ guanine in (A) standard mix and (B) oral cell sample from a smoker.

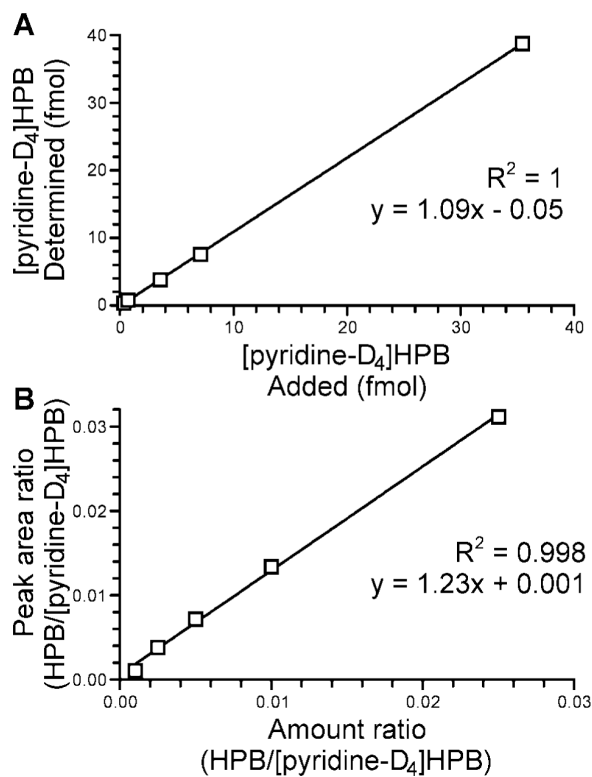


Figure 4.

(A) Relationship between added and measured [pyridine-D₄]HPB in the accuracy experiment. Various amounts of [pyridine-D₄]HPB (0.35, 0.7, 3.5, 7, and 35 fmol) were added to calf thymus DNA (50 pg), and analyzed by the method described in the text; (B) Linearity of HPB/[pyridine-D₄]HPB at constant [pyridine-D₄]HPB amount (4.8 fmol on-column) and HPB ranging from 0.005 to 0.12 fmol (on-column).

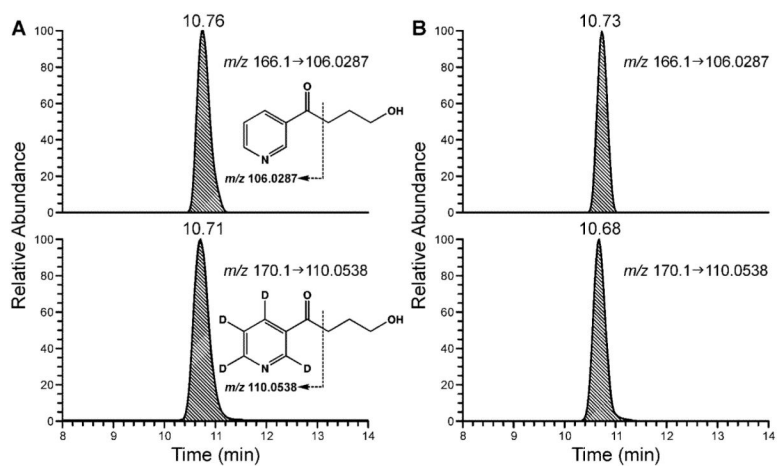


Figure 5. Typical accurate mass extracted ion chromatograms obtained upon analysis of HPB-releasing DNA adducts in human oral cells from a (A) smoker with HNSCC and (B) smoker without HNSCC.

Table 1

Levels of HPB-releasing DNA adducts in oral cells from smokers with HNSCC and smokers without HNSCC.

Smokers without HNSCC			Smokers with HNSCC		
subject #	DNA yield (µg)	HPB (pmol/mg DNA)	subject #	DNA yield (µg)	HPB (pmol/mg DNA)
1	1.24	LOQ ^a	1	0.96	0.59
2	0.21	LOQ	2	1.43	0.29
3	0.87	0.80	3	0.47	1.61
4	0.17	2.81	4	0.089	4.53
5	1.71	0.23	5	1.15	0.13
6	0.66	0.27	6	0.28	0.32
7	0.14	0.66	7	0.35	0.27
8	0.092	4.53	8	0.092	1.26
9	0.19	LOQ	9	0.14	2.56
10	0.27	0.68	10	2.46	0.13
11	0.48	0.56	11	0.063	29.8
12	2.25	0.028	12	0.057	31.8
13	0.068	2.68	13	8.71	0.10
14	0.11	0.37	14	0.035	92.2
15	0.021	LOQ	15	1.32	0.93
16	1.17	0.79	16	0.40	0.68
17	0.18	LOQ	17	0.087	22.9
18	0.23	LOQ	18	0.022	8.47
19	0.026	LOQ	19	0.23	2.33
20	0.006	47.0	20	0.48	1.47
21	0.78	0.68	21	0.49	LOQ
22	0.002	LOQ	22	0.038	3.90
23	0.60	LOQ	23	0.14	11.3
24	0.052	LOQ	24	0.33	17.9
25	0.050	LOQ	25	0.36	2.01
26	0.026	19.0	26	0.75	5.12
27	0.091	2.01	27	0.25	0.80
28	0.042	LOQ	28	0.052	1.55
29	0.039	LOQ	29	0.33	0.63
30	0.024	71.5	30	0.69	0.067
31	0.11	LOQ	Average^b	0.74	8.19
32	0.18	1.61	SD	1.57	17.8
33	0.10	0.11	Median	0.33	1.51
34	0.028	2.21	IQR	0.089, 0.69	0.32, 5.12
35	0.075	LOQ			
Average^b	0.35	4.53			

Smokers without HNSCC			Smokers with HNSCC		
subject #	DNA yield (μ g)	HPB (pmol/mg DNA)	subject #	DNA yield (μ g)	HPB (pmol/mg DNA)
SD	0.52	14.36			
Median	0.11	0.23			
IQR ^c	0.042, 0.48	0.001, 1.61			

^aLOQ, below the limit of quantitation (2 amol on-column)

^bTo calculate average values, 1/2 of the lowest level of HPB in the samples that HPB was quantifiable (0.028 pmol/mg DNA in subject 12 of smokers without HNSCC) was used for samples in which HPB levels were below LOQ. The value (0.028 pmol) was divided by its DNA yield (2.2 μ g), then multiplied by the DNA yield of samples with LOQ, and half of that value was used as the HPB levels in these samples.

^cIQR, inter-quartile range.

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