Simultaneous Solid-Phase Extraction and Gas Chromatographic–Mass Spectrometric Determination of Hemoglobin Adducts from Tobacco-Specific Nitrosamines and Aromatic Amines

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Abstract

A new method for the simultaneous determination of hemoglobin adducts from aromatic amines and tobacco-specific nitrosamines (TSNA) is described. After mild base-catalysed hydrolysis releasing aromatic amines and the TSNA adduct 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB), the extraction, cleanup, and concentration are performed by a one-step procedure using C_{18} cartridges. Determination in the picograms per gram of hemoglobin range by gas chromatography–mass spectrometry with negative chemical ionization requires a separate derivatization procedure with pentafluoropropionic anhydride and pentafluorobenzoylchloride for aromatic amines and HPB, respectively. The method is shown to be quantitative, reproducible, and applicable to the determination of hemoglobin adducts from monocyclic and bicyclic aromatic amines as well as TSNA in smokers and nonsmokers.

Introduction

Cigarette smoking has been shown to be a major risk factor for several types of human cancer. Because of their organospecificity in animal experiments, the tobacco-specific nitrosamines (TSNA) *N*'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), as well as the aromatic amine 4-aminobiphenyl (4-ABP), are suspected of being responsible for smoking-related cancer (1). Sidestream smoke often contains higher concentrations of these chemicals than mainstream smoke, raising the question of cancer induction by environmental tobacco smoke (2–4). Biological markers of the internal or effective dose of cigarette smoke constituents offer a significant advantage over air monitoring because they are able to register individual variability in the biological handling of these chemicals. Because of the observed correlation between protein and DNA binding of aromatic amines and TSNA, hemoglobin (Hb) adducts indicate that DNA damage may occur. Therefore, Hb adducts from 4-ABP and TSNA have been suggested as biomarkers of exposure in smokers and nonsmokers (5,6). Metabolic activation of NNN and NNK results in a common adduct releasing 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) through alkaline hydrolysis (6). For aromatic amines, the parent compounds are measured after hydrolysis (7). In the past, Hb has been measured separately for adducts of either TSNA or aromatic amines using liquid–liquid extraction procedures (7–13) or solid-phase extraction (12,14). It was the aim of this study to develop a method that would allow the analysis of different Hb adducts in a single sample of blood.

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Experimental

Chemicals

[D_9]-4-Aminobiphenyl (D_9-4-ABP) and [D_6]-butyrolactone were obtained from MSD Isotopes (Montreal, Canada). D_5-aniline, *o*-, m-, *p*-toluidine, 2,4-dimethylaniline, 2-ethylaniline, *o*-anisidine, 3-nitrobiphenyl, pentafluoropropionic anhydride (PFPA), and pentafluorobenzoylchloride (PFBC) were purchased from Aldrich (Steinheim, Germany). 4-Aminobiphenyl was obtained from Sigma (Deisenhofen, Germany). All other chemicals and solvents were of analytical grade and supplied by Merck (Darmstadt, Germany).

Trimethylamine (NMe_3) in hexane was prepared by adding 100 mg of trimethylamine hydrochloride to 9 mL of water and 1 mL 1 N NaOH and extracting in 10 mL hexane. The organic phase was kept dry over anhydrous sodium sulfate. All aqueous solutions were prepared with deionized water. To eliminate the possibility of background contamination, all glassware was rinsed twice with acetone and twice with chloroform.

[2,3,3,4,4-D_5]-4-hydroxy-1-(3-pyridyl)-1-butanone (D_5-HPB) and 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) were synthesized as described by Hecht et al. (15). 3-Aminobiphenyl (3-ABP) was synthesized by the catalytical reduction of 3-nitrobiphenyl with Sn/HCl.

Apparatus

The analysis of derivatized aromatic amines and HPB was performed by using gas chromatography–mass spectrometry

(GC–MS) with negative ion chemical ionization (NICI) in the selected ion monitoring (SIM) mode at 70 eV and a Carlo Erba QMD 1000 GC–MS (Fisons, Mainz, Germany). Methane and helium (at a flow rate of 2 mL/min) were used as reagent and carrier gases, respectively. The emission current was 300 µA, and the source temperature was 150°C. For maximum sensitivity, the mass spectrometer was tuned with Heptacos-FC 43 each day prior to analyses. To avoid interference of septum shavings, the septums were pretreated as follows: first the septums were soaked in toluene for 20 h, then they were heated under reflux in pentane for 10 min and kept in pentane for 20 h. Finally, the septums were dried for several days at 100°C.

Table I. Typical Retention Times of the Ions Monitored by GC–NICI–SIM–MS

Analyte	Ions of derivatives (*m/z*) PFPA	PFBC	Retention time (min)
Aniline	219		6.75
D_5-Aniline (internal standard)	224		6.73
o-Toluidine	233		7.25
m-Toluidine	233		7.62
p-Toluidine	233		7.74
2-Ethylaniline	247		7.80
2,4-Dimethylaniline	247		8.02
o-Anisidine	249		8.25
3-Aminobiphenyl	295		12.17
4-Aminobiphenyl	295		12.40
D_9-4-Aminobiphenyl (internal standard)	304		12.38
4-hydroxy-1-(3-pyridyl)-1-butanone		359	18.93
D_5-4-hydroxy-1-(3-pyridyl)-1-butanone (internal standard)		363	18.88

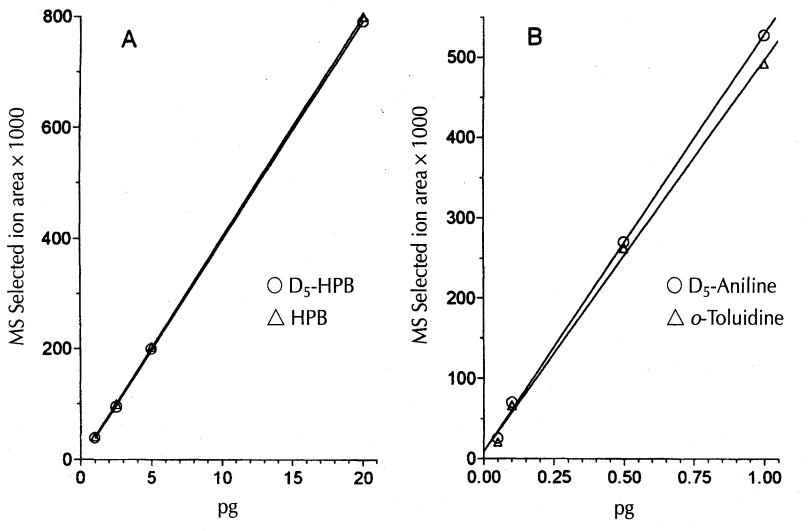

Figure 1. Representative calibration curves for the PFBC derivatives of the TSNA adduct HPB and its internal standard D_5-HPB (A) and for the PFPA derivatives of *o*-toluidine and its internal standard D_5-aniline (B) obtained by GC–NICI–SIM–MS; column: DB-5 MS (30 m x 0.25 mm, 0.25-µm film thickness); temperature program: 60°C held for 2 min, then programmed to 260°C at 20°C/min and held 4 min; carrier gas: helium at 2 mL/min; injection: 0.5–1 µL in the splitless mode with the valve open for 1 min; detection in the SIM mode at *m/z* values of 359 and 363 for HPB and D_5-HPB and at *m/z* values of 233 and 224 for *o*-toluidine and D_5-aniline, respectively, with dwell times of 150 ms.

The gas chromatograph was equipped with a 30-m x 0.25-mm-i.d. (0.25-µm film thickness) DB-5 MS column (J&W Scientific, Folsom, CA). The injector and interface temperatures were maintained at 220°C and 250°C, respectively. The initial oven temperature was 60°C for 2 min, followed by a temperature increase to 260°C at 20°C/min and a final hold at 260°C for 4 min. Up to 1 µL was injected in the splitless mode with the valve open for 1 min.

All ions were monitored with dwell times of 150 ms. For determination of aromatic amines, the ions of the $(M–HF)^-$ fragments of the PFPA derivatives were selected. The PFBC derivatives of HPB and D_5-HPB were detected by monitoring the ions of the $(M)^-$ and $(M–H)^-$ fragments, respectively. A list of all *m/z* values and typical retention times is given in Table I.

Methods

Twenty milliliters of blood from smoking and nonsmoking volunteers was drawn into tubes containing heparin to prevent blood clotting. The red blood cells (RBC) were separated by centrifugation at 4000 rpm for 10 min. After removal of the plasma, the RBC were washed three times with 0.9% saline and stored frozen at –20°C.

The erythrocytes were lysed by adding triple the volume of ice-cold deionized water. After 15 min, one-third of the volume of a 0.67M phosphate buffer (pH 6.6) was added, and the solution was mixed carefully. The lysate was centrifuged at 18,000 rpm for 30 min at 4°C to remove the cell debris. The supernatant was collected and dialyzed against deionized water at 4°C for two days (VISKING dialysis tubing 36/32; molecular weight cut-off, 12–19,000)(Serva, Heidelberg, Germany); the water was changed three times. The Hb solutions were frozen at –20°C until analysis.

The concentration of Hb in the solution was determined in duplicate 200-µL samples of Hb solution using Drabkin's Reagent (Sigma kit no. 525A). The absorbance was determined at 540 nm (UV-265FW spectrophotometer) (Shimadzu, Kyoto, Japan).

To duplicate samples of about 20 mL dialyzed Hb solution, 40-pg D_5-HPB, 40-pg D_9-4-ABP, and 80-pg D_5-aniline were added as internal standards. Two blank samples consisting of deionized water without addition of internal standards were run with each daily batch of samples. For hydrolysis of the adducts, one-tenth the volume of 1 N NaOH was added to each sample; the tubes were briefly vortex-mixed and sonically dispersed for 1 h at room temperature. The compounds of interest were isolated by solid-phase extraction (SPE) using C_{18} extraction tubes (Bond Elut with stainless steel frits, 500-mg sorbent mass, 3-mL volume)(Varian GmbH, Darmstadt, Germany) and an Adsorbex Sample Preparation Unit (Merck, Darmstadt, Germany). The SPE columns were pre-equilibrated with two volumes of deionized water without prior solvation with methanol,

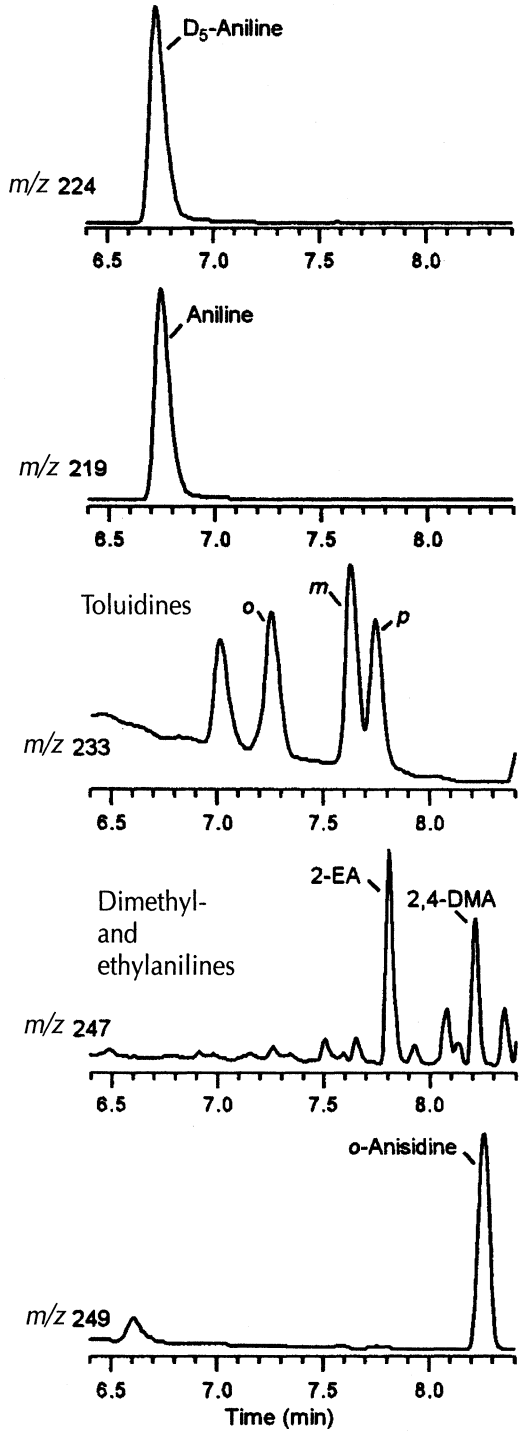

Figure 2. Selected ion detection mass spectral traces for the PFPA derivatives of monocyclic aromatic amines and the internal standard D_5-aniline obtained after alkaline hydrolysis of 390 mg hemoglobin from a smoker. Column: DB-5 MS (30 m x 0.25 mm, 0.25-µm film thickness); temperature program: 60°C held for 2 min, then programmed to 260°C at 20°C/min and held 4 min; carrier gas: helium at 2 mL/min; injection: 1 µL in the splitless mode with the valve open for 1 min; detection in the SIM mode at *m/z* values of 224 for D_5-aniline (76 pg), 219 for aniline (141 pg), 233 for *o*-, *p*-, and *m*-toluidine (64, 63, and 45 pg), 247 for 2-ethyl- and 2,4-dimethylaniline (8.5 and 5.7 pg), and 249 for *o*-anisidine (25 pg), with dwell times of 150 ms.

which did not improve the recovery. Vacuum (10–15 mm Hg, 1.3–2 kPa) was applied to pass the samples through the SPE columns. The columns were washed with two volumes of deionized water. The vacuum was turned off before the columns ran dry to avoid contamination by air pulled through the columns. To remove residual water, the columns were centrifuged at 3500 rpm for 10 min. The compounds of interest were eluted with 1–1.5 mL chloroform into glass tubes by centrifugation at 1500 rpm for 10 min. For separate derivatization of the aromatic amines and HPB with PFPA and PFBC, respectively, two equal parts of the chloroform eluate were transferred into autosampler vials (Zinsser Analytic, Frankfurt, Germany).

For derivatizing the aromatic amines, 1 µL PFPA, 5 µL NMe_3 in hexane, and 0.5 mL hexane were added to the chloroform eluate. After 15 min at room temperature, the solvents were evaporated using a vacuum centrifugal concentrator (Bachofer, Reutlingen, Germany), and the residue was dissolved in 20 µL hexane for analysis by GC–MS.

Prior to the derivatization of HPB, the chloroform was removed in the vacuum concentrator. We added 0.5 mL chloroform and 0.5 mL NMe_3 in hexane to the residue, followed by 1 µL of PFBC. The vial was shaken gently and then allowed to stand for 2 h at 60°C. The solvents were evaporated to dryness in the vacuum concentrator, and the residue was redissolved in 100 µL hexane and transferred into conical inserts for autosampler vials. After removing the solvent in the vacuum concentrator, the residue was finally dissolved in 5–10 µL hexane.

Aliquots of 0.5 µL for the aromatic amines and 1 µL for HPB

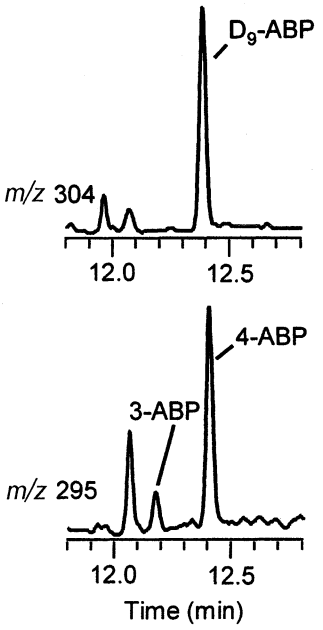

Figure 3. Selected ion detection mass spectral traces for the PFPA derivatives of 3- and 4-ABP and the internal standard D_9-4-ABP obtained after alkaline hydrolysis of 390 mg hemoglobin from a smoker. Column: DB-5 MS (30 m x 0.25 mm, 0.25-µm film thickness); temperature program: 60°C held for 2 min, then programmed to 260°C at 20°C/min and held 4 min; carrier gas: helium at 2 mL/min; injection: 1 µL in the splitless mode with the valve open for 1 min; detection in the SIM mode at *m/z* values of 304 for D_9-4-ABP (72 pg) and 295 for 3- and 4-ABP (2.5 and 14 pg, respectively), with dwell times of 150 ms.

were injected into the GC–MS system and analyzed under the conditions described above.

To determine the amount of each substance in the sample, the integrated peak area of the relevant ion was divided by the peak area of the corresponding internal standard ion and then multiplied by the ratio of the molecular weights of the respective substances. This value was divided by the grams of hemoglobin in the sample to calculate the adduct level expressed as picograms of substance per gram of hemoglobin. The reproducibility was studied by performing duplicate analyses on a series of five blood samples collected from one volunteer.

Results and Discussion

The aim of our study was to develop a new method to simultaneously determine hemoglobin adducts in human blood samples stemming from two classes of tobacco-related carcinogens: aromatic amines and tobacco-specific nitrosamines (1). Our method is based on the previously published methods for the separate determination of the TSNA-derived adduct HPB (9) and the aromatic amine 4-ABP (7). From the monocyclic aromatic amines reported to be simultaneously measurable with 4-ABP (8), we have selected the most significant smoking-related species (16). *o*-Anisidine was included because it has been released together with its precursor, *o*-nitroanisole, in large amounts into the environment due to an industrial accident in Germany in 1993 (17). Our method has already been applied successfully for the measurement of blood samples from smokers, nonsmokers, and users of nasal snuff (18,19). What follows is a discussion of the modifications to the existing methods.

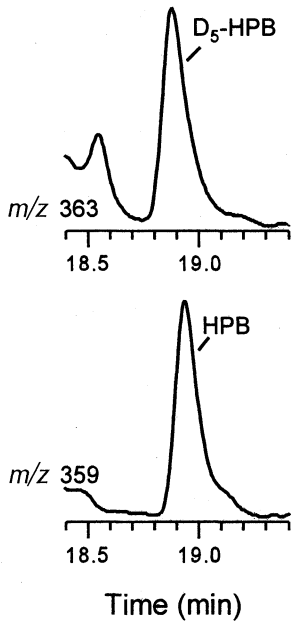

Figure 4. Selected ion detection mass spectral traces for the PFBC derivatives of the TSNA adduct HPB and its internal standard D_5-HPB obtained after alkaline hydrolysis of 390 mg hemoglobin from a smoker. Column: DB-5 MS (30 m × 0.25 mm, 0.25-µm film thickness); temperature program: 60°C held for 2 min, then programmed to 260°C at 20°C/min and held 4 min; carrier gas: helium at 2 mL/min; injection: 1 µL in the splitless mode with the valve open for 1 min; detection in the SIM mode at *m/z* values of 363 for D_5-HPB (1.8 pg) and 359 for HPB (1.6 pg), with dwell times of 150 ms.

Isolation of hemoglobin and adduct hydrolysis

These procedures were not modified. They were discussed in detail by Skipper and Stillwell (11). However, it should be pointed out that the type of water that is used is very critical. Because of significant contamination with aromatic amines, neither water from a Millipore Reagent System (Millipore, Königstein, Germany) nor commercial HPLC water (Lichrosolv, Merck) was suitable. Under our laboratory conditions, deionized tap water without further distillation did not introduce any background noise, with the exception of aniline. It is critically important to analyze water blanks together with every batch of analyses to control background noise.

Extraction and cleanup

After alkaline hydrolysis of the Hb solutions, the internal standards D_9-aminobiphenyl, D_5-aniline, and D_5-HPB were added prior to extraction. The deuterated internal standards of the aromatic amines are commercially available. We did not have access to standard hemoglobin solutions containing known amounts of D_9-aminobiphenyl adducts (11). However, because of the high reproducibility of our method, this modification is not believed to be critical. As the internal standard for the TSNA adduct releasing HPB, we preferred to synthesize D_5-HPB starting from commercially available D_6-butyrolactone (15) rather than D_2-HPB (9,20) because the molecular weight difference between the analyte and the internal standard should be as large as is practical (21).

The greatest modification of the existing methods was the replacement of multiple liquid–liquid extractions and, in the case of HPB, additional HPLC cleanup by a single SPE procedure. During development of the procedure, several steps turned out

Table II. Repeatability of Hemoglobin Adduct Determination*

Analyte	Amount (pg/g Hb) Mean	SD	RSD† (%)
Aniline	532.5	17.3	3.3
o-Toluidine	189.3	2.6	1.4
m-Toluidine	350.3	40.7	11.6
p-Toluidine	198.8	8.5	4.3
2-Ethylaniline	10.0	1.8	18.3
2,4-Dimethylaniline	12.0	1.4	11.8
o-Anisidine	58.8	5.1	8.6
3-Aminobiphenyl	1.4	0.3	20.7
4-Aminobiphenyl	7.5	1.3	17.2
4-hydroxy-1-(3-pyridyl)-1-butanone	4.0	0.5	12.4

* Duplicate analyses of blood samples from a nonsmoker were performed on five separate days within six weeks.
† RSD = relative standard deviation.

to be critical in order to avoid significant losses or cross-contamination. After alkaline hydrolysis and the addition of the internal standards, the Hb solution was drawn without any pH adjustment through SPE columns with stainless steel frits instead of the usual polyethylene frits. This prevented a major loss of the analytes and ensured that the solutions could be passed easily through the SPE columns. The flow rate of the applied sample was relatively high (> 1 mL/min) and, in general, extraction efficiencies are considered to be independent of flow rate. It is mandatory to prevent the columns from running dry on the vacuum manifold. For elution of the analytes, the columns have to be removed from the manifold and dried by centrifugation. Otherwise, cross-contamination and the introduction of aromatic amines from laboratory air cannot be prevented. Elution of the analytes with chloroform or ethylacetate resulted in the best recoveries. Again, the vacuum manifold had to be avoided. As an alternative to centrifugation, the organic solvent could also be pushed through by positive pressure with a disposable syringe adapted to the top of the column.

Derivatization and determination by GC–MS

For derivatization of aromatic amines (7) and HPB (9), different procedures were necessary that are not compatible. Therefore, after the elution from the SPE columns, the extracts were divided into two equal parts. Aromatic amines can be derivatized directly in the chloroform extract. This is important to avoid loss of the volatile amines during concentration. In contrast, no loss of PFPA derivatives of aromatic amines or of HPB itself occurred during concentration to dryness in the vacuum concentrator.

The high sensitivity necessary for determination of Hb adducts at environmental and passive smoke exposure levels requires determination of the analytes in the SIM mode.

Detection limit, recovery, and repeatability

The sensitivity of the GC–MS detector in the NICI mode is subject to daily variations. The instrument must be tuned every day to achieve maximum sensitivity. Therefore, it is not possible to determine absolute detection limits. Quantitative determination is only possible through the use of internal standards.

For the aromatic amines, the detection limits under negative ion chemical ionization conditions were about 50 fg for each $(M–HF)^-$ ion with a signal-to-noise ratio of 5:10. Recovery of the internal standards is generally about 50% for D_5-aniline and greater than 80% for D_9-4-ABP. This is within the range of the 40% aniline recovery reported by Stillwell et al. (8) and better than the 20–40% recovery of 4'-Fluoro-4-ABP reported by Bryant et al. (7). Linear response curves were obtained for PFPA derivatives of each amine in the range of 50 fg to 5 pg (Figure 1). The response factor for all monocyclic aromatic amines versus D_5-aniline on a molar basis was 1. For 3-ABP and 4-ABP when D_9-4-ABP was used as the internal standard, this factor had the same value.

In the case of HPB, the response of the mass spectrometer was linear in the range used for sample analysis (Figure 1). The response factor for HPB versus D_5-HPB was about 1. The detection limit for the PFBC derivative of HPB was approximately 200 fg, and the recovery of the internal standard was in the range of 30–50%, which is somewhat better than the 26% ± 14 reported by Carmella et al. (9).

In 10 mL of whole blood, reproducible detection of 0.6–2 pg/g Hb of the aromatic amines and about 2.5 pg/g Hb of HPB was possible when determined simultaneously. The deviation of duplicate analyses was between 5 and 15% for all substances. Typical traces obtained from a blood sample of a smoker are shown in Figures 2–4.

The precision and accuracy of the method were determined by repeated analysis of a 100-mL blood sample from a nonsmoker. Within six weeks, five 20-mL aliquots were taken for duplicate analyses on different days (Table II).

Reduction of background contamination

During the development of the analytical procedure, background contamination was the most serious problem. The reduction of multiple cleanup steps to one single solid phase extraction was essential. Through the use of disposable labware, cross-contamination could be prevented. Finally, water blanks carried through the procedure with each daily batch of 4–5 analyses gave no background signals in the GC–MS traces except for aniline (up to 30% of measured values). Occasionally, low background signals (less than 10% of measured values) are obtained for other monocyclic aromatic amines. Unavoidable contamination with aniline due to unknown sources has also been reported by Stillwell et al. (8) and by Sabbioni and Beyerbach (13). A relatively high background noise for HPB was reported by Carmella et al. (9), which we have also observed when using their HPLC cleanup step.

Conclusion

To our knowledge, the analytical procedure presented here is the first one allowing the simultaneous determination of Hb adducts from two classes of chemical carcinogens: aromatic amines and tobacco-specific nitrosamines. It has been successfully applied to biomonitoring in smokers, nonsmokers, and users of nasal snuff. The simplicity and accuracy of this method will make it possible to routinely monitor Hb adducts of these tobacco-related carcinogens.

Acknowledgment

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