Biomarkers, 2011; 16(3): 212–221

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2010.544755

informa
healthcare

RESEARCH ARTICLE

Analysis of 4-aminobiphenyl in smoker's and nonsmoker's urine by tandem mass spectrometry

Tiffany H. Seyler, and John T. Bernert

Emergency Response and Air Toxicants Branch, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA, USA

Abstract

The aromatic amine 4-aminobiphenyl (4-ABP) is present in tobacco smoke. In humans, it is also a known bladder carcinogen. We describe here a method for the quantification of total 4-ABP in urine using capillary gas chromatography/tandem mass spectrometry, with an effective detection limit in urine samples of approximately 0.87 pg/mL. We also examined the efficiency of chemical or enzymatic hydrolysis of urinary aromatic amine metabolites. Although we found acidic or basic hydrolysis effective, we found enzymatic hydrolysis (β-glucuronidase with either *Escherichia coli* or *Helix pomatia*) ineffective. As part of this work, we also confirm the presence of N-acetyl-4-ABP and 4-ABP glucuronide in human urine samples from smokers. These metabolites have been reported in animal studies, but previously they have not been identified in human samples. These metabolites, however, were found to be unstable and thus infeasible for biomonitoring. The final validated urinary total 4-ABP assay was applied to the analysis of samples from smokers and nonsmokers, whose status was confirmed from cotinine EIA measurements. Among 41 confirmed nonsmokers, the geometric mean (95% CI) of 4-ABP concentration was 1.64 pg/mg creatinine (1.30–2.07). Conversely, in 89 smokers, the geometric mean of 4-ABP concentration was significantly greater, at 8.69 pg/mg creatinine (7.43–10.16), $p < 0.001$. Our results indicate that following tobacco smoke exposure, total urinary 4-ABP is a reliable biomarker for exposure to this carcinogen.

Keywords: Chemical carcinogenesis, tobacco science, mass spectroscopy

Introduction

Largely because of exposure to certain aromatic amines present in tobacco smoke, cigarette smoking is an important risk factor for bladder cancer in humans (Bryant, et al. 1988, Castelao, et al. 2001, Fennell, et al. 2000, Vineis 1994, Yu, et al. 1994). But the risk is not confined to smokers. Second-hand tobacco smoke (SHS) is mainly composed of gases and particles generated from a mixture of sidestream smoke emitted from burning cigarettes between puffs and from exhaled mainstream smoke. SHS has been designated as a class A carcinogen and has been linked to a number of adverse health effects in exposed nonsmokers, including a potentially increased risk of bladder cancer (Hammond, et al. 1993, Riffelmann, et al. 1995, Silverman, et al. 1992, Skipper, et al. 2003, Teass, et al. 1993, Vineis 1994). SHS is a significant public health concern (DHHS 2006). Thus, both smoking and exposure to SHS are regarded as major sources of exposure for several aromatic amines in humans (Bryant, et al. 1987, Hammond, et al. 1993, IARC 1987, Silverman, et al. 1992, Vineis 1994).

Aromatic amines present in tobacco smoke include aniline, *ortho*-toluidine, 2-aminonapthylene and both 3- and 4-aminobiphenyl (ABP). Aniline is the predominant aromatic amine in cigarette smoke. Aniline, however, is a widespread compound in the environment. As a

Address for correspondence: Tiffany H. Seyler, Emergency Response and Air Toxicants Branch, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, 4770 Buford Highway, Mailstop F-47, Atlanta, GA 30341, USA. Tel.: (770) 488-4527, Fax: (770) 488-0181. E-mail: tvh2@cdc.gov

(Received 30 September 2010; revised 30 November 2010; accepted 30 November 2010)

consequence, concentrations measured in smokers and nonsmokers tend to be similar (Schnell 1994, Skipper & Tannenbaum 1994, Stillwell, et al. 1987). This nonspecificity restricts aniline's usefulness as a biomarker for tobacco smoke exposure. O*rtho*-toluidine is also known to be increased in smokers. It is relatively prominent among aromatic amines in tobacco smoke, but has multiple exposure sources and limited specificity for tobacco. Conversely, 4-ABP, although less concentrated in tobacco smoke, has few alternative environmental sources and is therefore moderately specific for tobacco smoke exposure.

4-ABP can be measured in blood as a hemoglobin adduct; concentrations of the 4-ABP hemoglobin adducts in smokers and nonsmokers have been shown to be significantly different (Castelao, et al. 2001, Dallinga, et al. 1998, Jiang, et al. 2007, Ketelslegers, et al. 2009, Mendes, et al. 2009, Richter & Branner 2002, Skipper, et al. 2003, Stillwell, et al. 1987). But the relation of the urinary concentrations of 4-ABP to smoking and tobacco smoke exposure has been more controversial. For example, Grimmer et al. (2000) found no difference in urinary 4-ABP concentrations between smokers and nonsmokers. Riedel et al. (2006) reported urinary 4-ABP levels in smokers approximately four times higher than in nonsmokers, and Sarkar et al. (2010) reported a significant difference in urinary aromatic amines between active smokers who continued to smoke and those who replaced cigarettes with a smokeless product, Marlboro Snus. In addition, unlike 4-ABP in blood—in which hemoglobin adduct formation is reasonably well understood (Bryant, et al. 1987, Hammond, et al. 1993, Stillwell, et al. 1987)—the intermediate metabolism of urinary 4-ABP has not been established. Talaska and Al-Zoughool (2003) and Riedel et al. (2006) have suggested a metabolic pathway for 4-ABP in which the toxicant is converted to *N*-acetyl or glucuronide forms in the liver and subsequently eliminated via renal excretion (Figure 1). To the best of our knowledge, however, no 4-ABP metabolites have been positively identified in human urine.

We describe here a reliable method for quantifying total 4-ABP in urine by using gas chromatography–tandem mass spectrometry (GC-MS/MS). We applied this method for measuring total 4-ABP in urine collected from smokers and nonsmokers. In addition, we have used both LC-MS/MS and GC high-resolution MS to confirm the presence of 4-ABP-glucuronide and *N*-acetyl-4-ABP in human urine samples from cigarette smokers.

Materials and methods

Standards and reagents

Native (unlabeled) 4-ABP was purchased from Aldrich (Milwaukee, WI). The isotopically labeled 4-ABP-D_9 internal standard was obtained from Toronto Research Chemicals (TRC, Toronto, Canada). Native *N*-acetyl-4-ABP and *N*-acetyl-4-ABP-D_3 were synthesized in-house by the method of Johnson et al. (1980). Correct structures were confirmed by using a Hewlett-Packard GC/MS and a Thermo-Finnigan MAT95 high-resolution mass spectrometer, interfaced to a Hewlett-Packard 5890 gas chromatograph. The mass fragmentation obtained by both low- and high-resolution GC/MS was consistent with the expected structure of *N*-acetyl-4-ABP; the mass accuracy obtained during high-resolution MS was 0.6 ppm. This compound was further

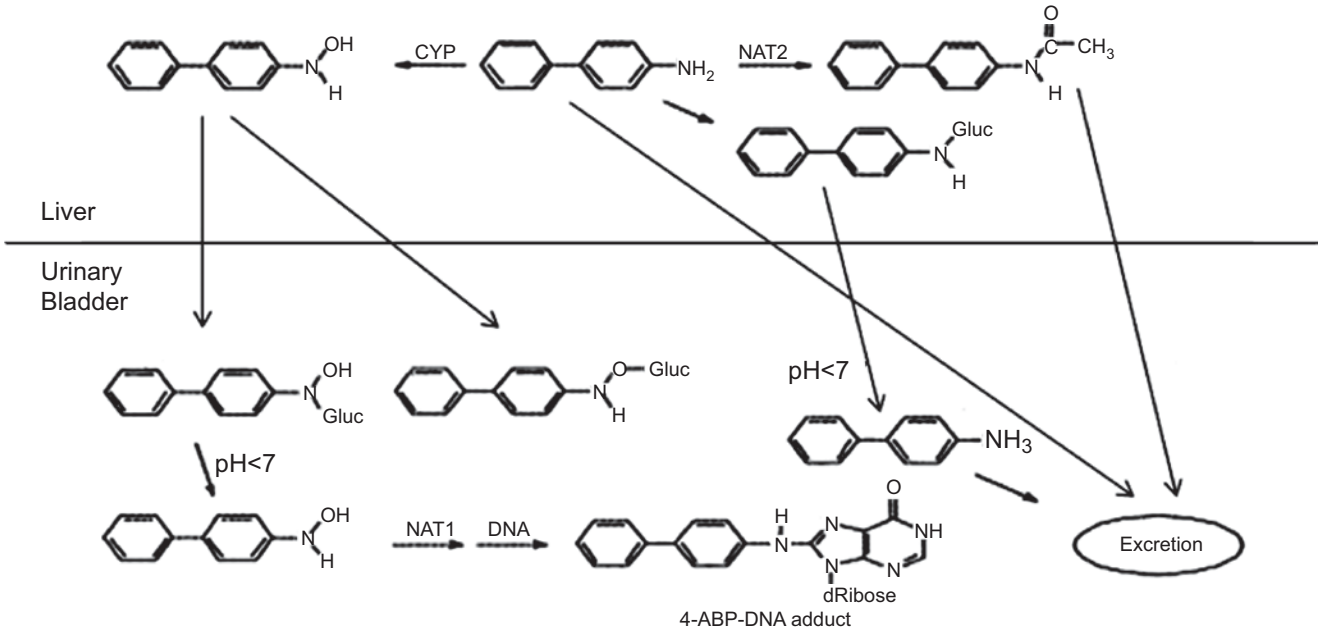

Figure 1. Proposed metabolic pathway of renal excretion of 4-ABP in human according to Riedel et al (2006).

confirmed by analyzing the 4-ABP content following NaOH hydrolysis. 4-ABP-glucuronide was purchased from TRC. High-purity hydrochloric acid, pentafluoropropionic acid anhydride (PFPA), saccharic acid-1,4-lactone and trimethylamine were obtained from Aldrich. Solvents were GC^2 grade except for water, which was HPLC grade. All solvents were purchased from Burdick and Jackson Labs (distributed by VWR, Suwanee, GA), and all gases were ultra-high purity grade. Clean Screen® ETG 200 mg Carbon cartridges were purchased from United Chemical Technology, Inc. (Bristol, PA). Solid phase extraction (SPE) HLB® (hydrophilic-lipophilic-balanced) and Certified C18® cartridges were purchased from Waters (Milford, MA). ChemElut® liquid-liquid extraction and sodium sulfate cartridges were obtained from Varian (Walnut Creek, CA).

Samples

Urine samples were obtained from Tennessee Blood Services Corp. (Memphis, TN). All samples were collected anonymously, although information on race, age and self-reported smoking status was recorded. Samples were collected once randomly during the day, excluding first morning void. Forty samples from 40 heavy smokers (at least one pack of cigarettes per day) were used in an initial study in which we analyzed for free and total 4-ABP and the identification of 4-ABP-glucuronide and *N*-acetyl-4-ABP. An additional 171 urine samples were used in a follow-up study for the quantification of total urinary 4-ABP. They included 24 white female smokers, 8 white female nonsmokers, 25 white male smokers, 15 white male nonsmokers, 25 black male smokers, 24 black male nonsmokers, 25 black female smokers and 25 black female nonsmokers. The average age of all participants was 35.6 ± 11.5. The results of these 171 samples were normalized to creatinine and further evaluated. Cotinine measurements were made by using a commercial EIA test kit (Neogen Corporation, Lansing, MI). We used a urine cotinine cutoff value of 50 ng/mL to distinguish between smokers and nonsmokers (Benowitz, et al. 2002).

Instrumentation

We conducted assays of total urinary 4-ABP using a Thermo Scientific TSQ Quantum mass spectrometer equipped with a Trace™ gas chromatograph and the Xcalibur data system (Thermo Scientific, San Jose, CA). The GC was equipped with a programmable temperature vaporization (PTV) injector. The on-column injection system incorporated a 0.52-mm by approximately 2.0-m deactivated fused silica retention gap connected to a J&W DB-17 capillary column (0.25 mm × 30 m × 0.25 μm film thickness) (Agilent, Wilmington, DE). A Glas-Col© rugged rotator was used for all liquid-liquid extraction (Glas-Col LLC, Terre Haute, IN). We performed concentration steps with a Vacuum Evaporator Savant SpeedVac AES 200 equipped with a VP190 high vacuum pump, an RT 4104 refrigerated trap and a VaporNet VN100 auxiliary trap (Thermo Scientific/Savant Instruments, Farmingdale, NY).

Analyses of 4-ABP-glucuronide and *N*-acetyl-4-ABP metabolites were conducted on an API 4000 mass spectrometer equipped with an Agilent 1100 HPLC and the Analyst data system (Applied Biosystem, Foster City, CA). The LC column was an XTerra MS C18 (4.6 × 150 mm, 3.5 μm) (Waters, MA). For confirmation of the *N*-acetyl-4-ABP metabolite, we used a Thermo-Finnigan MAT95 high-resolution mass spectrometer, interfaced to a Hewlett-Packard 5890 gas chromatograph.

Preparation of urinary total 4-ABP samples

Urine samples were shipped frozen and on receipt were stored at –70°C. Frozen samples were allowed to thaw, and two water blanks were included at this point. To a 5 mL aliquot of each sample, we added 500 pg of internal standard followed by 50 μL of 10 M NaOH (freshly prepared and allowed to cool to room temperature before use). The samples were hydrolyzed for 15 h at 90°C. After hydrolysis, the samples were cooled and extracted with 8 mL of hexane for 1 h using a rugged rotator at 60 rpm (Glas-Col LLC). If an emulsion formed, it was broken by freezing at –70°C for approximately 1–2 h. The hexane layer was then transferred, back-extracted with 1 mL of 0.1 N HCl, and neutralized with 10 M NaOH. We used Waters HLB columns to clean the samples further. HLB cartridges were conditioned with 1 mL methanol and 1 mL water, followed with a 1 mL water wash after sample loading, and the analytes were eluted with 2 mL of methylene chloride. The methylene chloride eluant was collected through sodium sulfate cartridges to eliminate residual water. The final eluant was dried in the Savant to approximately 300 μL of residual volume.

Analytes were derivatized in methylene chloride to their pentafluoropropionates by adding 3 μL of 0.1 M trimethylamine (TMA-HCl prepared in water and extracted with hexane) followed by 2 μL of PFPA. Samples were mixed and allowed to stand at room temperature for 30 min. The solution was then evaporated in an amber vial with a 300-μL insert, and 10 μL of toluene was added to the residue. In most cases, samples were analyzed immediately. If necessary, however, vials could be sealed at this point and stored at –20°C for several days before analysis.

Analysis of total urinary 4-ABP by GC MS/MS

We compared a gradient and a constant temperature injection (80–250°C vs. 250°C), and we found no difference in sensitivity using the splitless mode. Thus, the PTV injector was kept at 250°C for the entire GC run. The injection mode was PTV splitless with surge. The transfer line temperature was maintained at 210°C, and the ion source temperature was 250°C. A 1-μL aliquot of the

derivatized sample in toluene was injected with an initial oven temperature of 80°C. The oven temperature was then ramped to 180°C at a rate of 40°C/min, then to 270°C at a rate of 60°C/min, with a hold at the final temperature for 1.5 min. The column was a J&W DB-17 capillary column (0.25 mm × 30 m × 0.25 μm film thickness) (Agilent, Wilmington, DE). The He carrier gas was maintained at a constant flow of 1.2 mL/min. The mass spectrometer was operated in the EI positive ion multiple reaction monitoring (MRM) mode with a scan time of 0.050 s. The main transition ion of the native unlabeled 4-ABP was *m/z* 315 → 168, which represents the loss of PFP from the pentafluoropropionic derivative, and m/z 324 → 177, for a similar loss from the deuterated internal standard ion. Collision gas was ultra-pure Argon at 1.2 m Torr, and the collision energy was 30 eV. Normal run times were about 6.5 min.

The on-column limit of detection (LOD) for 4-ABP was 0.24 pg, estimated using the extrapolated, limiting standard deviation obtained from the calibration curves. The effective LOD, however, was calculated as approximately 0.87 pg/mL in urine samples, based on the standard deviation from multiple analysis of the low-QC samples prepared from nonsmokers' urine. The overall recovery was approximately 20–25%.

Preparation of urinary 4-ABP metabolite samples

During development of the method for measuring total 4-ABP in urine, we also examined samples for the presence of suspected additional metabolites. We collected samples to be measured for 4-ABP-glucuronide analyses. Then we added 1 mL of 0.5 M saccharic acid-1,4-lactone per 5 mL of urine as a specific inhibitor of β-glucuronidase (Zenser, et al. 1999). This was to inhibit the metabolism by either bacterial or human enzymes present in urine samples. Samples were either processed immediately or frozen at –70°C, and then processed on ETG, HLB or C18 cartridges. When we used ETG cartridges, 50 μL formic acid was added to each urine sample (1 mL) and loaded onto the cartridge, which had been preconditioned with 2 mL of 1% formic acid. The cartridge was washed with 2 mL water, dried at approximately 10 mm Hg for 10 min, and then eluted with 2 mL of 1% formic acid in methanol. For HLB or C18 cartridges, 3 mL samples were loaded onto the cartridges, which had been preconditioned with 1 mL methanol and 1 mL water, then washed twice with 1 mL aliquots of water and finally eluted twice with 2 mL aliquots of 20% ethanol in methylene chloride. ChemElut 1 mL cartridges were preconditioned with KOH followed by methylene chloride, and oven-dried at 55°C for at least 24 h before use. Analytes were eluted from ChemElut columns with two 5 mL aliquots of methylene chloride. In all cases, the final eluant was dried in the Savant and reconstituted with 10 μL of 40:60 water:methanol.

Analysis of 4-ABP metabolites by LC-ESI MS/MS

4-ABP-glucuronide and N-acetyl-4-ABP were analyzed by LC-electrospray ionization/MS-MS (LC-ESI/MS-MS). The ionspray needle voltage was 5.5 kV, and the source temperature was maintained at 650°C; collision energy was set to 17–30 eV. The LC column compartment was kept at 45°C, and the injection volume was 5 μL. For 4-ABP-glucuronide analysis, the initial eluant was 98% A (5 mM ammonium acetate containing 0.05% ammonium hydroxide) and 2% B (methanol). It was changed to 100% B in a linear gradient over a period of 6.5 min and held for 5 min. The flow rate was held at 1.00 mL/min. For N-acetyl-4-ABP, the initial eluant was 95% A and 5% B. It was changed to 95% B in a linear gradient over a period of 2 min and held for 5 min. The flow rate was held at 0.80 mL/min.

Further analysis of N-acetyl-4-ABP by GC high-resolution MS

N-acetyl-4-ABP and *N*-acetyl-4-ABP-D_3 were analyzed by GC high-resolution MS using a MAT 95 instrument (Thermo-Finnigan). The analytical column was a J&W DB-1701 capillary column (0.25 mm × 30 m × 0.25 μm film thickness) (Agilent, Wilmington, DE). The temperature program was 18°C/min from 80°C to 270°C/min with a hold at the final temperature for 1.5 min. The source was held at 240°C, and the accelerating voltage was 4700 V. The resolution was set to a minimum of 10,000 operating in lock-mass mode.

Additional methods

Urinary cotinine was measured by an enzyme-linked immunoassay using a commercial kit manufactured by Neogen Corporation. Urinary creatinine measurements were made using a commercial Creatinine Plus kit (Roche Diagnostics, Indianapolis, IN).

Method validation

Method validation was carried out as follows:

1. *Precision*. Intermediate precision from at least two pools at different levels from multiple runs over a period of at least 1 week with $N \geq 5$ for each pool was obtained. The CV for each level was then calculated and accepted only if it was ≤10%.
2. *Short-term precision*. At least six repetitive injections of a low, a medium and a high standard were made, and the SD and CV were calculated.
3. *Accuracy*. If precision was acceptable, assay blank urines spiked with analyte at low, mid and high range levels was performed. Each spiked pool was analyzed at least three times, and the bias in the results as: (observed – expected)/expected × 100% was calculated.
4. *Blanks*. A series of blanks through the entire procedure were analyzed and determined for any interference.
5. *Linearity*. Data from the accuracy evaluation was used

to ensure the linearity of the results over the concentration range.

6. *Carryover*. Runs in which high and low samples were intermixed with low samples and/or blanks following immediately after the high samples were carried out. This arrangement was maintained both during cleanup and during MS analysis, and carryover under these conditions was assessed.
7. *Stability*. (i) A well-mixed pool was prepared and assayed at least in duplicate. Over a period of about a week, this pool was frozen, re-thawed and reanalyze. The effect of freeze-thaw cycles on the results was evaluated. (ii) Residual frozen aliquots from a series of analyses was re-assayed over a period of days, and the storage stability of these processed samples was determined.
8. LOD/LOQ. An initial LOD was estimated from series of standard injections ($N=6$); and the final reported LOD was estimated from the variance of a low pool ($N=20$).
9. *Robustness*. A control pool was analyzed for ruggedness by varying: (i) amount of 10 M NaOH used in hydrolysis: 150, 200 and 250 μL; (ii) time of hexane liquid-liquid extraction after the hydrolysis: 1, 2 and 3 h; and (iii) the amount of PFPA: 1, 2 and 3 μL.
10. QA/QC. Final characterization (at least 20 replicates over at least 10 days) for the QC pools to be used in the studies was conducted, and the standard deviation was evaluated using a SAS program.

Calculations

For total 4-ABP measurements, we prepared calibration curves from the area ratios of the native and labeled compounds. We used 14 standards ranging from 0.00 to 25 pg/μL, which we assayed in duplicate before each analytical series. The lowest nonzero standard was 0.05 pg/μL, and the concentration of the 4-ABP-D_9 internal standard was 50 pg/μL. To confirm instrument performance before and after each run, at least two standards were analyzed daily.

Each sample run included 10–40 unknowns, 2 blanks, 1 cartridge blank and 2 quality control (QC) pools, one high and one low, prepared from smoker and nonsmoker urine pools, respectively. The initial sample concentrations were calculated by comparing the ion ratios to the standard curve. Final reported concentrations were derived by subtracting the value for the blank (if any) and then normalizing to the creatinine concentration. If the blank exceeded 0.4 pg, runs were rejected. Runs were also rejected if any QC pool was outside the three standard deviation limits for that pool, or if both of the QC pools were outside of two standard deviation limits in the same direction. Quality assurance for these runs followed a multirule algorithm implemented in SAS/QC as previously described (Caudill, et al. 2008).

Results

Analysis of 4-ABP in urine

4-ABP appears in urine in both free and bound forms. Thus during the development of the total 4-ABP analysis, we investigated three different hydrolysis conditions: acidic with 1 mL of 37% HCl for 1 h at 80°C (Riedel, et al. 2006); basic with 50 μL of 10 M NaOH for 1, 3 and 15 h at 90°C; and an enzymatic hydrolysis (Grimmer, et al. 2000) for 16 h at 37°C with β-glucuronidase (*Escherichia coli* type IX-A). In samples incubated with the enzyme for between 5 and 8 h, the amount of 4-ABP was slightly higher. But no additional increases were found during further incubations from 8 to 48 h, and the total 4-ABP recovered was much less than that obtained following chemical hydrolysis. In general, for urine samples, the enzymatic hydrolysis under these conditions was found less effective than chemical hydrolysis. Acidic hydrolysis for 1 h and basic hydrolysis for 3 h had about the same efficiency. Although acidic hydrolysis required a shorter time, we observed a higher background and extensive interference in *N*-acetyl-4-ABP LC/ESI/MS-MS mass spectral analysis. After 9–15 h, samples spiked with 50–500 pg *N*-acetyl-4-ABP were completely hydrolyzed under basic conditions. Yields were significantly less among the 40 smoker samples hydrolyzed under basic conditions for 1–3 versus 15 h ($p<0.01$). Therefore, all reported results were obtained by using basic hydrolysis for 15 h.

Free and total 4-ABP were quantified by positive GC/EI-MS-MS using MRM mode. Short-term precision was estimated by the repetitive analysis of three levels of standards with concentrations of 0.3, 12.5 and 25.0 pg/μL. A single run with six replicates each resulted in relative standard deviations of 6.7%, 4.6% and 1.7%, respectively. Interday precision was estimated over a period of 2 weeks, with daily analyses of the two QC pools of low and high levels. Three replicates at each level were analyzed each day for 7 days, with an observed day-to-day precision of 7.5 and 8.4% for low and high levels, respectively. Accuracy was examined by using fortified QC samples at three levels of 5.0, 25.0 and 125 pg/mL. The average experimental values from nine independent runs were ±4, 7 and 10% of expected values, respectively. Carryover was examined by two runs using three samples of blank, low and high QC. QC with high level of analytes was followed by either low level or blank samples. Four replicates of the blank samples were first analyzed to establish a baseline to determine analyte levels independent of carryover. Carryover was then examined by comparing successive pairs of samples (high followed by low or high followed by blank). No carryover could be detected. Therefore, the carryover limit was defined as the analyte concentration in the highest standard. Finally, a control pool was analyzed for ruggedness by varying (i) the amount of 10 M NaOH used in hydrolysis (150, 200 or 250 μL), (ii) the time of hexane liquid-liquid extraction after the

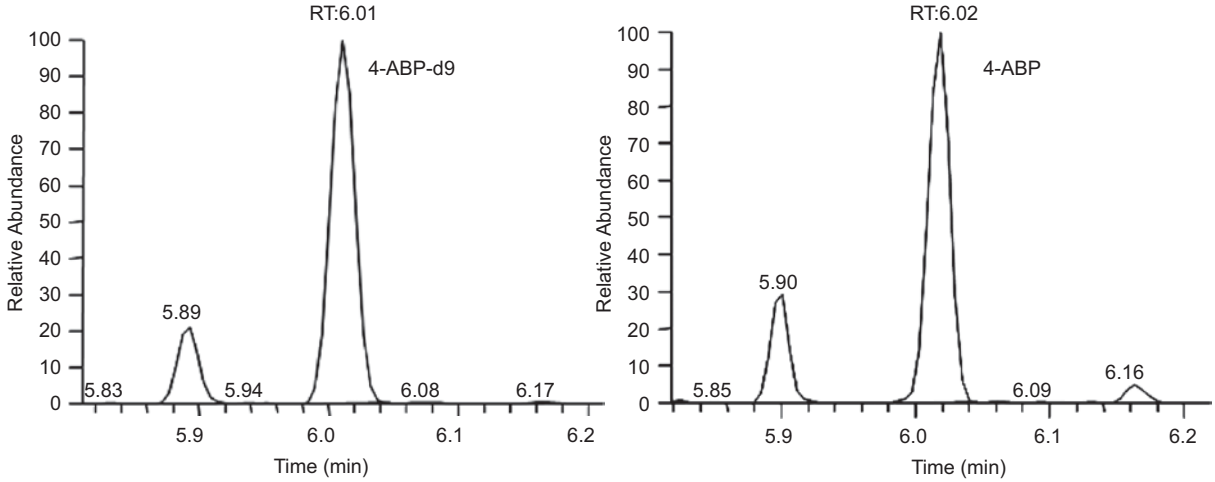

Figure 2. Chromatograms of 4-ABP (20.9 pg/mL) and 4-ABP-D_9 in a smoker urine sample.

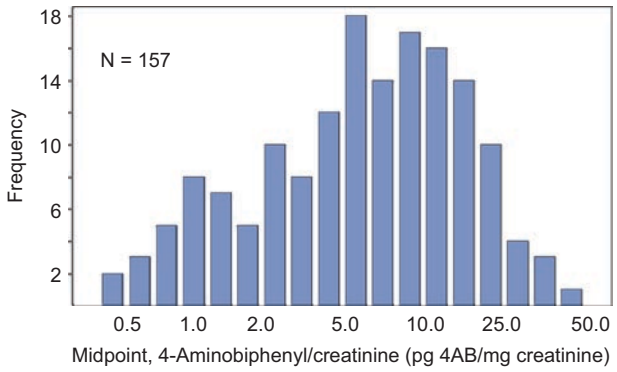

Figure 3. Log distribution of total 4-ABP concentration normalized to creatinine, of all subjects with detectable 4-ABP level with one extreme outlier excluded on the high side.

hydrolysis (1, 2 or 3 h) and (iii) the amount of PFPA (1, 2 or 3 μL). The observed variations had no significant effect on the analyte's calculated values. To ensure method performance, we analyzed two QC pools, one low and one high prepared from nonsmoker and smoker urine, respectively, to assess long-term stability and present assay sensitivity. The results of 29 runs acquired during a 3-month period showed no significant deviations or trends, consistent with both analyte and method stability during that time. For the QC low pool, the mean value was 4.26 pg/g creatinine and the standard deviation was 0.875. For QC high pool, the mean value was 29.4 pg/g creatinine and the standard deviation was 5.170.

The optimized method was then applied to the analysis of a set of 171 urine samples from both smokers and nonsmokers. Figure 2 shows MRM chromatograms of 4-ABP and 4-ABP-D_9 in a smoker sample. Figure 3 shows the distribution of total 4-ABP concentrations (free plus hydrolyzed), normalized to creatinine, of all subjects with detectable 4-ABP level with one extreme outlier excluded on the high side. The distribution has two relative maxima with an approximate separation at about 2 pg/mg creatinine. Although there is a clear overlap between the two regions, we find that when using a cut-point of 2 pg/mg creatinine with this group, those with corrected urinary concentrations of ≥2 included 70% smokers based on self-report. Those below that concentration included only 16% self-reported smokers. Among the 89 smokers in this group whose smoking status was confirmed by urinary cotinine EIA analysis, the geometric mean (and 95% CI) concentration of total 4-ABP was 8.69 pg/mg creatinine (7.43–10.16 pg/mg creatinine). Among the 41 nonsmokers whose smoking status was also confirmed by urinary cotinine EIA analysis, the geometric mean total 4-ABP level was significantly lower, at 1.64 pg/mg creatinine (1.30–2.07 pg/mg creatinine), $p < 0.001$.

Examination of 4-ABP metabolites

To better understand the nature of the metabolites of 4-ABP present in human urine, we also analyzed for two predicted 4-ABP metabolites (4-ABP-glucuronide and *N*-acetyl-4-ABP) using the additional methods described earlier. To determine the best extraction recovery of 4-ABP and its metabolites, an evaluation was made of different SPE cartridges, including ETG Carbon, HLB, ChemElut and certified Sep-Pak C18 cartridges. When saccharic acid-1,4-lactone was not present, the recovery of 4-ABP-glucuronide on all cartridges was quite poor. Saccharic acid-1,4-lactone served as an inhibitor for endogenous bacteria and human β-glucuronidase enzyme activity. Therefore, in all experiments investigating the presence of the glucuronides, samples were collected with 1 mL of 0.5 M saccharic acid-1,4-lactone. Still, recoveries on the ETG Carbon, ChemElut and C18 cartridges remained poor, even in the presence of the inhibitor (fewer than 5%). The best recoveries were with the HLB cartridges, approximately 32% with inhibitor present and 5% without the inhibitor. The recovery of *N*-acetyl-4-ABP was about the same using either the HLB cartridge

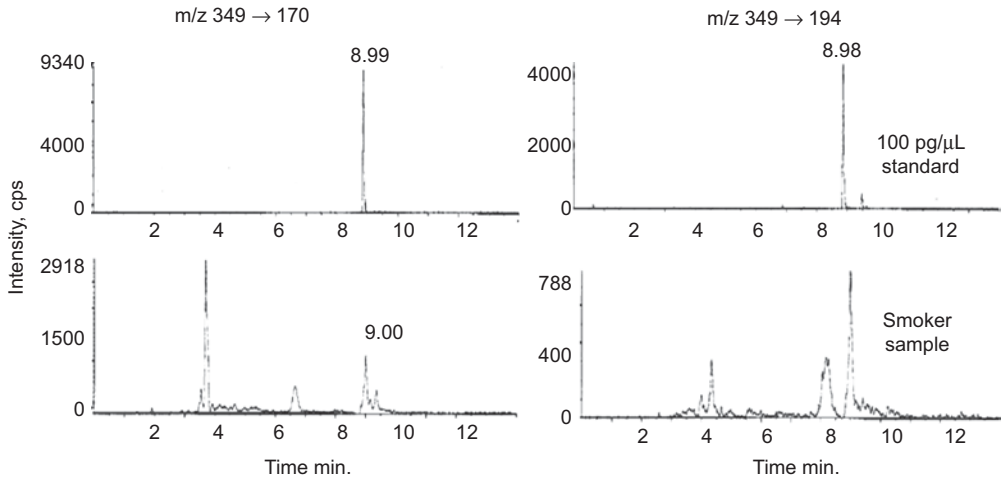

Figure 4. HPLC-ESI/MS-MS-extracted ion chromatogram of 4-ABP-glucuronide from a 100 pg/μL standard and a smoker urine sample.

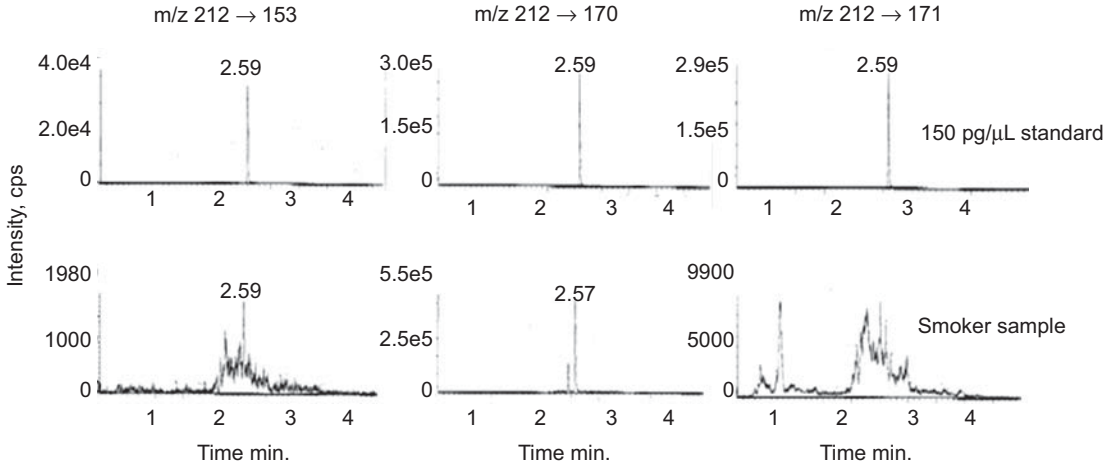

Figure 5. HPLC-ESI/MS-MS-extracted ion chromatogram of *N*-acetyl-4-ABP from a 150 pg/μL standard and a smoker urine sample. The ISTD in samples is not stable for quantization analysis.

or following direct extraction with methylene chloride. To confirm the recovery efficiency, all residual samples were hydrolyzed, derivatized and analyzed on GC/MS-MS for total 4-ABP after the initial LC analyses. Unless indicated otherwise, all the results were obtained by using HLB cartridge extractions.

Figure 4 shows an extracted ion chromatogram using HPLC-ESI MS/MS of 4-ABP-glucuronide from a 100 pg/μL standard and a smoker urine sample. 4-ABP-glucuronide was detected in 7 of the 40 samples from smokers collected while using 1 mL of 0.5 M saccharic acid-1,4-lactone. Three of those seven samples contained *N*-acetyl-4-ABP, which was also detected in four additional samples. Figure 5 shows the LC of *N*-acetyl-4-ABP from a 150 pg/μL standard and a smoker urine sample. The presence of *N*-acetyl-4-ABP in these smoker samples was further confirmed by using capillary GC linked to a high-resolution magnetic sector mass spectrometer. Figure 6 shows the TIC and exact mass of *N*-acetyl-4-ABP in a 5 pg/μL standard (A) and a smoker urine sample extract (B) analyzed on the latter instrument, both with 0.6 ppm mass accuracy. The level of free 4-ABP observed in these 40 smoker urine samples ranged from a nominal zero to 9.96 pg/mL. The geometric means of free and total 4-ABP concentration are 1.1 and 15.3 pg/mL, respectively.

Table 1 lists the collision energy and ion transitions for the analysis of 4-ABP and its glucuronide and acetate metabolites. Transitions of 4-ABP-glucuronide were monitored at m/z 349 →170, 349 →194. The presence of *N*-acetyl-4-ABP was monitored by using the transition ions m/z 212 →170, 212 →153, and m/z 215 →171 for the D_3 deuterated internal standard. The quantitation-to-confirmation ion ratio is approximately 6.24. For 4-APB analysis, the main transition ion of the native unlabeled 4-ABP was m/z 315 → 168, which represents the loss of PFP from the pentafluoropropionic derivative, and m/z 324 → 177, for a similar loss from

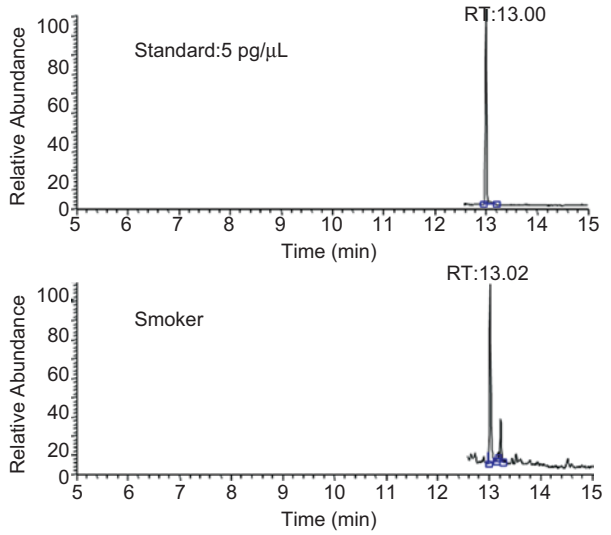

Figure 6. GC/MS total ion chromatogram and the exact mass spectra of *N*-acetyl-4-ABP from a 5 pg/µL standard and a smoker urine sample. Both high mass spectra were obtained with 0.6 ppm accuracy, 212.0993 amu.

Table 1. Collision energy (CE) and m/z transitions for the analysis of 4-ABP-glucuronide and *N*-acetyl-4-ABP by LC API MS/MS, and of 4-aminobiphenyl by GC MS/MS.

Analyte	CE	m/z	Instrument
4-ABP-glucuronide	17	349/170	LC/MS-MS
4-ABP-glucuronide	25	349//194	LC/MS-MS
N-acetyl-4-ABP	25	212/170	LC/MS-MS
N-acetyl-4-ABP	35	212/153	LC/MS-MS
N-acetyl-4-ABP-D_3 (-CD_3)	25	215/171	LC/MS-MS
4-Aminobiphenyl-PFPA	30	315/168	GC/MS-MS
4-Aminobiphenyl-D_9-PFPA	30	324/177	GC/MS-MS

the deuterated internal standard ion. Consequently, the area ratio of these two signals was used in the quantification of 4-ABP.

Discussion

We have developed and validated a method for the measurement of 4-ABP in human urine samples, and we have examined the nature of the metabolite forms of 4-ABP that may be present. 4-ABP-glucuronide was detected in 7 of 40 urine samples from smokers when the samples were collected with 1 mL saccharic acid-1,4-lactone present as an inhibitor for bacteria and human β-glucuronidase enzyme. An inhibitor was necessary for this latter measurement, and all standards and samples had to be prepared and analyzed within the same day because of the instability of 4-ABP-glucuronide. It has been suggested that spontaneous conversion of 4-ABP-glucuronide to free 4-ABP may take place under acidic conditions (Riedel, et al. 2006, Talaska & Al-Zoughool 2003) which may have contributed to its detection in only some samples. Yet in these 40 samples we did not observe a notable pH dependency. The pH of seven samples that contained 4-ABP-glucuronide ranged from 5.0 to 6.3, whereas no 4-ABP-glucuronide appeared in four samples with a pH above 7.0. Our results do confirm that in some cases 4-ABP-glucuronide is formed and can be detected. But its lability suggests that conversion of the glucuronide to the free form for total measurements of 4-ABP should be readily achieved.

N-acetyl-4-ABP was also detected in seven samples, three of which contained 4-ABP-glucuronide. We attempted to quantify *N*-acetyl-4-ABP in the urine, but we found that during sample preparation the internal standard was not stable. The D_3 standard was synthesized in-house with the deuterium on the acetyl group (*N*-methyl D_3), but during analysis we observed extensive proton exchange with the amine group, resulting in an observed transition ion at m/z 215 →171 instead of 215 →170. Thus, although we were able to confirm the presence of *N*-acetyl-4-ABP in at least some of the smoker samples, this internal standard instability precluded the reliable quantification of this metabolite. Nevertheless, we were able to confirm the hydrolysis of both the glucuronides and the acetate under our analytical conditions, and we conclude that the measurement of total urinary 4-ABP following their hydrolysis is the preferred approach.

We found that GC and MS/MS can reliably measure total 4-ABP concentrations in urine samples. In this study, the concentrations were significantly higher among smokers than in nonsmokers, which is consistent with previous observations from the analysis of 4-ABP adducts in blood (Seyler, et al. 2010). When classified on the basis of urinary cotinine concentrations, the smokers' mean concentration of 4-ABP was approximately five times higher than in nonsmokers (8.69 pg/g creatinine vs. 1.64 pg/g creatinine; $p < 0.001$). These results are in substantial agreement with Riedel et al. (2006), in which the concentration of urinary 4-ABP in smokers was reported as approximately 4.5 times greater than in nonsmokers. This significant difference might be expected based on the known content of 4-ABP in cigarette smoke, including significant concentrations in SHS. In fact, nonsmokers exposed to SHS may be at particular risk given that many aromatic amines—including 4-ABP—are enriched severalfold in sidestream relative to mainstream tobacco smoke. Thus our results confirm Riedel's, indicating that total urinary 4-ABP could be a useful biomarker for tobacco exposure in a manner similar to 4-ABP measurement in blood. And monitoring urinary 4-ABP could be useful, given that urine samples are relatively easy to collect, the collection process is noninvasive, and this matrix should be more amenable than blood samples to biomonitoring in large studies and in infants or young children. Still, these two matrices might also be expected to reflect different exposure periods. 4-ABP hemoglobin adducts are quite stable over time, whereas urinary analytes typically have a relatively short elimination half-life. The choice of matrix might be conditioned in part on the time-frame of interest.

We believe that this study confirmed for the first time the presence of 4-ABP-glucuronide and *N*-acetyl-4-ABP in human urine. Furthermore, under our analytical conditions standards of both of these metabolites were found to be completely hydrolyzed. The resulting total urinary 4-ABP levels were quite stable, and were shown to be significantly different between smokers and nonsmokers. Thus for identifying people exposed to aromatic amines from tobacco smoke—either through active use or from exposure to SHS—urinary total 4-ABP is a potentially valuable marker.

Acknowledgements

We thank Connie Sosnoff and Kay DeLong (Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention) for assistance with the urine cotinine immunoassay analysis, and Pamela G. Olive (Clinical Chemistry Branch, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention) for assistance with the creatinine measurements. We also thank Dr. Ram Jain for his statistical analysis.

Declaration of interest

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention. Use of trade names and commercial sources is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services or the Centers for Disease Control and Prevention.

References

Benowitz N, Jocob PI, Ahijevych K, Jarvis M, Hall S, LeHouezec J, Hansson A, Lichtenstein E, Henningfield J, Tsoh J, Hurt R, Velicer W. (2002). Biochemical verification of tobacco use and cessation. *Nicotine Tob Res* 4:149–159.

Bryant MS, Skipper PL, Tannenbaum SR, Maclure M. (1987). Hemoglobin adducts of 4-aminobiphenyl in smokers and nonsmokers. *Cancer Res* 47:602–608.

Bryant MS, Vineis P, Skipper PL, Tannenbaum SR. (1988). Hemoglobin adducts of aromatic amines: associations with smoking status and type of tobacco. *Proc Natl Acad Sci usa* 85:9788–9791.

Castelao JE, Yuan JM, Skipper PL, Tannenbaum SR, Gago-Dominguez M, Crowder JS, Ross RK, Yu MC. (2001). Gender- and smoking-related bladder cancer risk. *j Natl Cancer Inst* 93:538–545.

Caudill SP, Schleicher RL, Pirkle JL. (2008). Multi-rule quality control for the age-related eye disease study. *Stat Med* 27:4094–4106.

Dallinga JW, Pachen DM, Wijnhoven SW, Breedijk A, van 't Veer L, Wigbout G, van Zandwijk N, Maas LM, van Agen E, Kleinjans JC, van Schooten FJ. (1998). The use of 4-aminobiphenyl hemoglobin adducts and aromatic DNA adducts in lymphocytes of smokers as biomarkers of exposure. *Cancer Epidemiol Biomarkers Prev* 7:571–577.

DHHS (U.S. Department of Health and Human Services). 2006. The health consequences of involuntary exposure to tobacco smoke: a report of the Surgeon General. Atlanta: U.S. Department of Health and Human Services, CDC, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health.

Fennell TR, MacNeela JP, Morris RW, Watson M, Thompson CL, Bell DA. (2000). Hemoglobin adducts from acrylonitrile and ethylene oxide in cigarette smokers: effects of glutathione S-transferase T1-null and M1-null genotypes. *Cancer Epidemiol Biomarkers Prev* 9:705–712.

Grimmer G, Dettbarn G, Seidel A, Jacob J. (2000). Detection of carcinogenic aromatic amines in the urine of non-smokers. *Sci Total Environ* 247:81–90.

Hammond SK, Coghlin J, Gann PH, Paul M, Taghizadeh K, Skipper PL, Tannenbaum SR. (1993). Relationship between environmental tobacco smoke exposure and carcinogen-hemoglobin adduct levels in nonsmokers. *J Natl Cancer Inst* 85:474–478.

IARC. (1987). IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans.

Jiang X, Yuan JM, Skipper PL, Tannenbaum SR, Yu MC. (2007). Environmental tobacco smoke and bladder cancer risk in never smokers of Los Angeles County. *Cancer Res* 67:7540–7545.

Johnson HJ Jr, Cernosek SF Jr, Gutierrez-Cernosek RM, Brown LL. (1980). Development of a radioimmunoassay procedure for 4-acetamidobiphenyl, a metabolite of the chemical carcinogen 4-aminobiphenyl, in urine. *J Anal Toxicol* 4:86–90.

Ketelslegers HB, Godschalk RW, Eskens BJ, Dallinga JW, Gottschalk RW, van Schooten FJ, van Delft JH, Kleinjans JC. (2009). Potential role of cytochrome P450-1B1 in the metabolic activation of 4-aminobiphenyl in humans. *Mol Carcinog* 48:685–691.

Mendes P, Liang Q, Frost-Pineda K, Munjal S, Walk RA, Roethig HJ. (2009). The relationship between smoking machine derived tar yields and biomarkers of exposure in adult cigarette smokers in the US. *Regul Toxicol Pharmacol* 55:17–27.

Richter E, Branner B. (2002). Biomonitoring of exposure to aromatic amines: haemoglobin adducts in humans. *J Chromatogr B Analyt Technol Biomed Life Sci* 778:49–62.

Riedel K, Scherer G, Engl J, Hagedorn HW, Tricker AR. (2006). Determination of three carcinogenic aromatic amines in urine of smokers and nonsmokers. *J Anal Toxicol* 30:187–195.

Riffelmann M, Müller G, Schmieding W, Popp W, Norpoth K. (1995). Biomonitoring of urinary aromatic amines and arylamine hemoglobin adducts in exposed workers and nonexposed control persons. *Int Arch Occup Environ Health* 68:36–43.

Sarkar M, Liu J, Koval T, Wang J, Feng S, Serafin R, Jin Y, Xie Y, Newland K, Roethig HJ. (2010). Evaluation of biomarkers of exposure in adult cigarette smokers using Marlboro snus. *Nicotine Tob Res* 12:105–116.

Schnell FC. (1994). *Reviews in Environmental Toxicolgoy*. Vol. 5. ed. Hodgson E. Toxicology Communications, Inc.: Raleigh, NC:51–160.

Seyler TH, Reyes LR, Bernert JT. (2010). Analysis of 4-aminobiphenyl hemoglobin adducts in smokers and nonsmokers by pseudo capillary on-column gas chromatography-tandem mass spectrometry. *J Anal Toxicol* 34:304–311.

Silverman DT, Hartge P, Morrison AS, Devesa SS. (1992). Epidemiology of bladder cancer. *Hematol Oncol Clin North Am* 6: 1–30.

Skipper PL, Tannenbaum SR. (1994). Molecular dosimetry of aromatic amines in human populations. *Environ Health Perspect* 102 Suppl 6:17–21.

Skipper PL, Tannenbaum SR, Ross RK, Yu MC. (2003). Nonsmoking-related arylamine exposure and bladder cancer risk. *Cancer Epidemiol Biomarkers Prev* 12:503–507.

Stillwell WG, Bryant MS, Wishnok JS. (1987). GC/MS analysis of biologically important aromatic amines. Application to human dosimetry. *Biomed Environ Mass Spectrom* 14:221–227.

Talaska G, Al-Zoughool M. (2003). Aromatic amines and biomarkers of human exposure. *J Environ Sci Health c Environ Carcinog Ecotoxicol Rev* 21:133–164.

Teass AW, DeBord DG, Brown KK, Cheever KL, Stettler LE, Savage RE, Weigel WW, Dankovic D, Ward E. (1993). Biological monitoring for occupational exposures to o-toluidine and aniline. *Int Arch Occup Environ Health* 65:S115–S118.

Vineis P. (1994). Epidemiology of cancer from exposure to arylamines. *Environ Health Perspect* 102 Suppl 6:7–10.

Yu MC, Skipper PL, Taghizadeh K, Tannenbaum SR, Chan KK, Henderson BE, Ross RK. (1994). Acetylator phenotype, aminobiphenyl-hemoglobin adduct levels, and bladder cancer risk in white, black, and Asian men in Los Angeles, California. *J Natl Cancer Inst* 86:712–716.

Zenser TV, Lakshmi VM, Davis BB. (1999). Human and *Escherichia coli* beta-glucuronidase hydrolysis of glucuronide conjugates of benzidine and 4-aminobiphenyl, and their hydroxy metabolites. *Drug Metab Dispos* 27:1064–1067.