

Internal Exposure, Health Effects, and Cancer Risk of Humans Exposed to Chloronitrobenzene

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Chloronitrobenzenes (CNBs) are important intermediates for the production of dyes, pesticides, rubber chemicals, and drugs. 2CNB and 4CNB are possible human carcinogens. Therefore, it is important to develop methods to biomonitor people exposed to these occupational and environmental pollutants. We developed a method to determine hemoglobin (Hb) adducts of CNBs. Nitrobenzenes and the resulting arylamines yield the same sulfinamide adducts. Therefore, after base hydrolysis of the isolated Hb the corresponding arylamines are released and quantified by GC–MS. The method was applied to monitor 39 Chinese workers exposed to CNB and 15 control workers from the same factory. The determined Hb adduct levels were compared to the measured air levels, the clinical blood and urine parameters, and health effects identified in the workers. The median Hb adduct levels resulting from exposure to 2CNB and 4CNB were 82.9 and 1013 pg/mg of Hb, respectively. The median air concentrations determined from personal samplers were 0.37 and 0.87 mg/m³ for 2CNB and 4CNB, respectively. The air levels did not correlate with the Hb adduct levels. The median Hb adduct levels were higher in workers with fatigue, eye irritation, splenomegaly, and cardiovascular effects. Most negative urinary clinical parameters were present at higher median Hb adduct levels. The clinical blood parameters decreased at higher adduct levels. The daily dose was estimated from the Hb adduct levels and used to estimate the cancer risk.

Introduction

2-Chloronitrobenzene (2CNB) and 4-chloronitrobenzene (4CNB) are used primarily as chemical intermediates in the

production of dyes, pesticides, rubber chemicals, lumber preservatives, drugs, corrosion inhibitors, and photographic chemicals (1–3). Although these chemicals are solids at room temperature, the vapor pressures of these chemicals are sufficiently high to result in significant inhalation exposure (4). 2CNB and 4CNB are high production volume chemicals and are produced worldwide. There are 17 known companies producing 2CNB and 4CNB in China. Chloronitrobenzenes have repeatedly been reported in samples taken in the environment (5, 6). The U.S. Environmental Protection Agency (EPA) lists 4CNB as a possible human carcinogen, on the basis of no evidence in humans and positive evidence in mice (7). 2CNB induced tumors in different organs of rats and in the liver of mice (8, 9). The EPA established a cancer slope factor (upper limit on the lifetime probability that a cancer-causing chemical will cause cancer at a dose of 1.0 (mg/kg)/day) for 2CNB and 4CNB (7, 9). In contrast, the international agency for cancer research (10) determined that 2CNB and 4CNB are not classifiable as carcinogenic to humans (group 3) on the basis of an absence of data in humans and inadequate data in animals. No carcinogenicity studies have been performed for 3CNB. 2CNB and 4CNB were mutagenic in *Salmonella typhimurium* with S9 activation (11). In addition, both compounds induced sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells. In pharmacokinetic studies in male F344 rats, approximately 86–93% of a single oral dose of 4CNB was absorbed (1). In male F344 or Sprague–Dawley rats given a single oral radiolabeled dose, urinary excretion was the main route of elimination; at 72 h, recovery of label was 68–75% in urine and 12–21% in feces. In both studies, the highest amount of label in tissues was recovered in fat, whole blood/blood cells, and the spleen. The metabolism of 4CNB appears to be similar in humans and rats. Yoshida (12, 13) evaluated urinary metabolites in humans and rats exposed to 4CNB (Figure 1). Urine from eight male dock workers was collected for up to 29 days following an accidental exposure; absorption was considered to have occurred by inhalation and dermally (13). The main metabolites were *N*-acetyl-S-(4-nitrophenyl)-L-cysteine (48% of the total metabolites), 4-chloroaniline (29.9%), 2-chloro-5-nitrophenol (12.2%), and 2-amino-5-chlorophenol (8.7%). Nitrobenzenes are reduced in the gut by the microflora and in the liver by cytochrome P-450 enzymes (14). *N*-Hydroxyarylamines (Figure 1) are the putative genotoxic intermediates of arylamines and nitroarenes (15). *N*-Hydroxyarylamines react with DNA, tissue proteins, and blood proteins (16, 17). Hemoglobin adducts of arylamines and nitroarenes have been determined for several compounds (17, 18). *N*-Hydroxyarylamines are oxidized in the erythrocytes to the nitrosoarenes, which react with the thiol group of cysteine 93 of the β -chain of hemoglobin (Hb) to give semimercaptals (19). Elimination of hydroxide to the sulfenamide cation, which will partly allocate its positive charge to the less electronegative sulfur atom, and addition of a water molecule with subsequent proton rearrangement yields the sulfinamide (20). Hb adducts relate to an integrated dose over the lifetime of the erythrocyte since adduct formation is unhindered by active repair processes (21). In rats dosed with 2CNB, 3CNB, and 4CNB, Hb adducts were formed (22, 23). The corresponding arylamines 2-chloroaniline (2CA), 3CA, and 4CA were released on base hydrolysis of the Hb. The extent of hemoglobin binding, expressed as a hemoglobin-binding index [(mmol of compound/mol of hemoglobin)/(mmol of compound/kg of body mass)], was 2.1, 54.2, and 215.4 for 2CNB, 3CNB, and 4CNB, respectively. In animals given radiolabeled 4CA,

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To date, there have been no reports in the literature for Hb adducts in humans resulting from acute or chronic exposure to CNBs. Occupational and environmental health regulations favor measurement of the environmental concentrations (air–water, food measurements) as a proxy marker of exposure. Such measurements do not consider individual uptake and interindividual variation in metabolism of the compounds (17, 25–27). A better approach to establish a risk estimation for the exposed populations is the measurement of the internal dose (urine metabolites) or the biologically effective dose (protein adducts, DNA adducts). In general, urine metabolites reflect only the exposure levels of the last 48 h (25). Hemoglobin adducts reflect the exposure history of the red blood cells (ca. 120 days in humans). The goal of the present study was to develop a method to biomonitor workers exposed to CNBs. In addition, the Hb adduct levels were compared to the air measurements, clinical blood parameters, and health effects.

Anhydrous sodium sulfate (Na_2SO_4) and bovine hemoglobin (Bv Hb) were obtained from Fluka (www.sigmaaldrich.com). 2-Chloroaniline (99%) (2CA), 3-chloroaniline (99%) (3CA), 4-chloroaniline (99%) (4CA), 2,4-dichloroaniline (>99%) (24DCA), 2,6-dichloroaniline (>99%) (26DCA), 3,4-dichloroaniline (>99%) (34DCA), and $[\text{I}^{13}\text{C}]_4$ -4-chloroaniline (99%) ($[\text{I}^{13}\text{C}]_4\text{CA}$) were obtained from Promochem (www.lgcpromochem.com). Pentafluoropropionic anhydride (PFPA) was obtained from Supelco (www.sigmaaldrich.com). Unisolve pure *n*-hexane, sodium hydroxide (NaOH), and LiChrosolve pure H_2O were obtained from Merck (Darmstadt, Germany). The deuterated standard d_4 -2-methylaniline (d_4 -2MA) was synthesized according to the procedure of Sabbioni and Beverbach (28).

same week. Each participant was interviewed with a questionnaire about their general status, exposure history, smoking habits, previous medical record, and present symptoms. For the present study 40 (38 males) exposed workers and 17 (16 males) control workers could be recruited. The number of smokers in the exposed and control worker groups was 28 (70%) and 11 (65%), respectively. The mean age (\pm standard deviation) of the workers and of the control workers was 32 ± 8.4 and 39.2 ± 13.1 years, respectively. The mean number of work years in the present factory was 5.9 ± 3.5 and 11.4 ± 6.3 in exposed and control workers, respectively. The Medical Department of the Chinese Academy of Preventive Medicine performed the following examinations: (a) physical examinations (blood pressure, cardiovascular system, nervous system, heart rate); (b) routine blood tests (red blood cell (rbc) count, white blood cell (wbc) count, methemoglobin (methHb), serum glutamic pyruvic transaminase (sgpt)) and urine (pH, bilirubin, protein, wbc's, rbc's, glucose) tests; (c) electrocardiogram (ECG) (ECG1, sinus tachycardia, sinus bradycardia; ECG2, arrhythmia; ECG3, conduction); (d) ultrasonic type B examination for liver and spleen; (e) serological assays of hepatitis B antigens and antibodies, which were conducted because hepatitis B is rather common in China, and liver damage can also be caused by some of the nitroarenes.

Collection of Blood Samples. In total, 60 blood samples were collected from the CNB factory workers. Of these, 39 were from exposed workers and 15 were from nonmatched, controls working in the same factory. Six additional control blood samples were analyzed from the Chinese Academy of Preventative Medicine. Hb was isolated as described previously (29).

Hb Isolation and Hb Hydrolysis. Hb (20 mg) was dissolved in 0.1 M NaOH (1.5 mL) by vortex mixing in glass screw-capped tubes (22 mm \times 100 mm) with Teflon liners (Supelco, 15 mm). The hexane solution (10 μ L) with the recovery standards (1 ng [13 C]4CA, d_4 -2MA) was added to the basic Hb solution (pH > 12). After 1 h in a shaking bath at room temperature, *n*-hexane (3 mL) was added. The water/organic phases were vortex mixed for 1 min prior to phase separation by centrifugation at 2000g for 10 min. The samples were frozen in liquid nitrogen to facilitate efficient phase separation following thawing. The organic layer was dried through a pipet containing anhydrous Na₂SO₄ (1 g) and washed through with *n*-hexane (1.5 mL). The dried organic phase was collected in a graduated tapered tube (98 mm \times 17 mm). An aliquot of PFPA (2 μ L) was added to each sample and left at room temperature for 10 min to fully derivatize. The samples were evaporated carefully under a stream of nitrogen to approximately 200 μ L and then transferred to 200 μ L micro-inserts for autosampler vials (32 mm \times 12 mm) with vial springs. The samples were carefully evaporated to dryness under a stream of nitrogen, and the residue was taken up in ethyl acetate (15 μ L).

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TABLE 1. Retention Time and Major EI and NCI Mass Fragments Determined from Full-Scan Spectra, m/z 50–350, of the PFPA Derivatives of Arylamines^a

amine	retention time (min)	EI m/z^c (rel intens)	NCI m/z^b	internal standard	retention time (min)	EI m/z	NCI m/z
2CA	3.9	273 (26), 275 (9) 238 (100), 126 (30)	253, 255	d_4 -2MA ^d	4.1	281	237
3CA	5.0	273 (100), 275 (33), 154 (91), 111 (63)	253, 255	[¹³ C]4CA	5.1	281	261
4CA	5.1	273 (100), 275 (33) 154 (51), 126 (74)	253, 255	[¹³ C]4CA	5.1	281	261

^a The presented ions were chosen for single-ion monitoring of arylamines in Hb extracts of workers exposed to mixed chloronitrobenzene.

^b These ions correspond to the molecular ion minus hydrofluoride, $[M - HF]^-$. ^c m/z 273 and 275 correspond to the positively charged molecular ion, $[M]^+$. ^d This was used as an internal standard for 2CA in NCI mode. In EI mode 2CA was related to [¹³C]4CA.

derivatives analyzed are described in the Results and Discussion (Table 1). For electron impact ionization (EI), the electron energy was 70 eV, the emission current was 300 μ A, and the source temperature was 200 °C. For negative chemical ionization (NCI), with methane as the reagent gas, the source pressure was 100 eV, the emission current was 300 μ A, and the source temperature was 200 °C. Additional sensitivity and specificity were obtained by monitoring individual ions for each of the analytes. These are described in the Results and Discussion (Table 1). The dwell time for analyte masses m/z 237, 253, 255, 261, 271, and 287 was 40 ms. Additionally, confirmatory spectra were obtained by scanning all masses from m/z 50 to m/z 350 with the chromatographic conditions described below.

Quantitation of Hb Adducts by GC–MS and Method Validation. For determination of 2CA–Hb cleavage products, concentrations of authentic 2CA standard, 0, 0.5, 1.0, 5.0, and 10.0 ng of 2CA/20 mg of BvHb, were used, and for 4CA, concentrations of authentic 4CA standard, 0, 1.0, 10.0, 25.0, and 50 ng of 4CA/20 mg of BvHb, were used. In addition, d_4 -2MA (2 ng/20 mg of BvHb) and [¹³C]4CA (1 ng/20 mg of BvHb) were added as internal standards for 2CA and 4CA, respectively. The standards were taken through the same assay procedure as for the human Hb samples. Quantitation of 2CA and 4CA was determined from the abundance ratio of ions m/z 253 and 237 (m/z 261 for [¹³C]4CA) relative to a calibration line of known standards run on the same day of analysis. For each calibration line, the r value of the regression line was greater than 0.99 and indicated that the response of the mass analyzer, in NCI mode, was linear over the concentration range of 2CA and 4CA analyzed. The data were averaged values from two separate determinations. The difference between determinations ranged between 2.3% and 12.4% for 2CA and between 1.5% and 8.1% for 4CA.

The levels of 2CA and 4CA were also determined in EI mode. For each calibration line, the r of the regression line was greater than 0.99 and indicated that the response of the mass analyzer, in EI mode, was linear over the concentration range determined. The data points and error bars are averaged values \pm standard deviation determined from five independent replicates. The data were averaged values from two separate determinations. The difference in adduct levels between each determination, by GC–MS–EI, ranged from 2.8% to 12.3% for 2CA and from 1.2% to 12.2% for 4CA.

The interday variation was evaluated from the precision associated with replicate determinations of Hb samples from three exposed 4CNB workers, analyzed over the course of 4 weeks. The values presented (pg/mg of Hb) were an average from eight separate determinations \pm the standard deviation. The values determined for 2CA and 4CA were 68.4 ± 6.4 and 613.6 ± 42.1 pg/mg of Hb (worker no. 01), 81.6 ± 8.4 and 1291 ± 72 pg/mg of Hb (worker no. 19), and 1.27 ± 0.075 and 1379 ± 37.8 pg/mg of Hb (worker no. 21), respectively.

The precision of the assay was evaluated from the standard deviation associated with determination of known amounts of 2CA and 4CA added to BvHb and taken through the assay procedure. Known amounts of standard solutions (five

repeats) were spiked into basic solutions of BvHb (20 mg) with internal standards. The values presented (ng/mg of Hb) were an average from five separate determinations \pm the standard deviation. The recovery of 2CA from 0.5 and 5 ng spikes was 0.46 ± 0.047 and 4.72 ± 0.35 ng of 2CA/mg of Hb, respectively. The recoveries of 4CA determined from 1 and 11 ng of 4CA/20 mg of Hb were 1.024 ± 0.03 and 10.69 ± 0.78 ng of 4CA/20 mg of Hb, respectively.

Air Monitoring. Air monitoring of 2CNB, 3CNB, and 4CNB was performed according to NIOSH method 2005 (www.cdc.gov/niosh/nmam/pdfs/2005.pdf). Personal exposure monitoring (19 workers) was carried out using personal samplers from SKC (www.skinc.com). The SKC pumps were precalibrated with two silica gel adsorbent tubes (SKC product no. 226-10) connected in parallel. The flow rate used was 200 mL/min. The samplers were attached to the lapels of the workers to monitor the breathing zone. Each set of tubes were exchanged after 2 h to prevent overloading and breakthrough of the adsorbed material. All silica tubes were desorbed (ca 1 h) in 1 mL of methanol (both front and back sections). Each sample (1 μ L) was analyzed by GC–FID (HP 9897): temperature program, 90 °C, increase at 10 °C/min to 200 °C; column, Ultra 1 (Agilent) (cross-linked methylsiloxane, 50 m \times 0.32 mm, 0.52 μ m film thickness). Calibration lines were constructed using authentic standards; correlation coefficients of >0.99 and detection limits of 5 μ g/mL (10 ng injected) were achieved.

Statistical Analyses. Statistical analyses were performed with the program SPSS 10.0. The results of the questionnaire and of the medical examination were not known to the scientists performing the Hb adduct analyses. All results were disclosed at the end of the analyses. All correlation coefficients (r) given in the text were determined with the Pearson correlation method. Similar correlation coefficients ($\pm 5\%$) were obtained with the Spearman rank order test (data not shown).

Results and Discussion

Quantitation of arylamines covalently bound to Hb was based upon the analytical procedure described by Sabbioni and Beyerbach (28). The PFPA derivatives of the parent arylamines, cleaved from the Hb, were separated by gas chromatography on a silica fused capillary column and detected by a coupled mass spectrometer. The arylamines were analyzed in both NCI and EI ionization modes. For increased sensitivity, the most abundant characteristic fragments, determined in the full scan spectra, were selected for single-ion monitoring. The fragments for each arylamine of interest are presented in Table 1.

Three arylamines were identified in organic extracts of hydrolyzed Hb, isolated from exposed workers. These arylamines were characterized by their mass fragments in both NCI and EI ionization modes and by their retention times with respect to those of authentic standards. In NCI mode the ion abundance of fragments $[M - HF]^-$ at m/z 253 and 255 was acquired for characterization of each arylamine. These fragments were observed at an abundance ratio of 3:1,

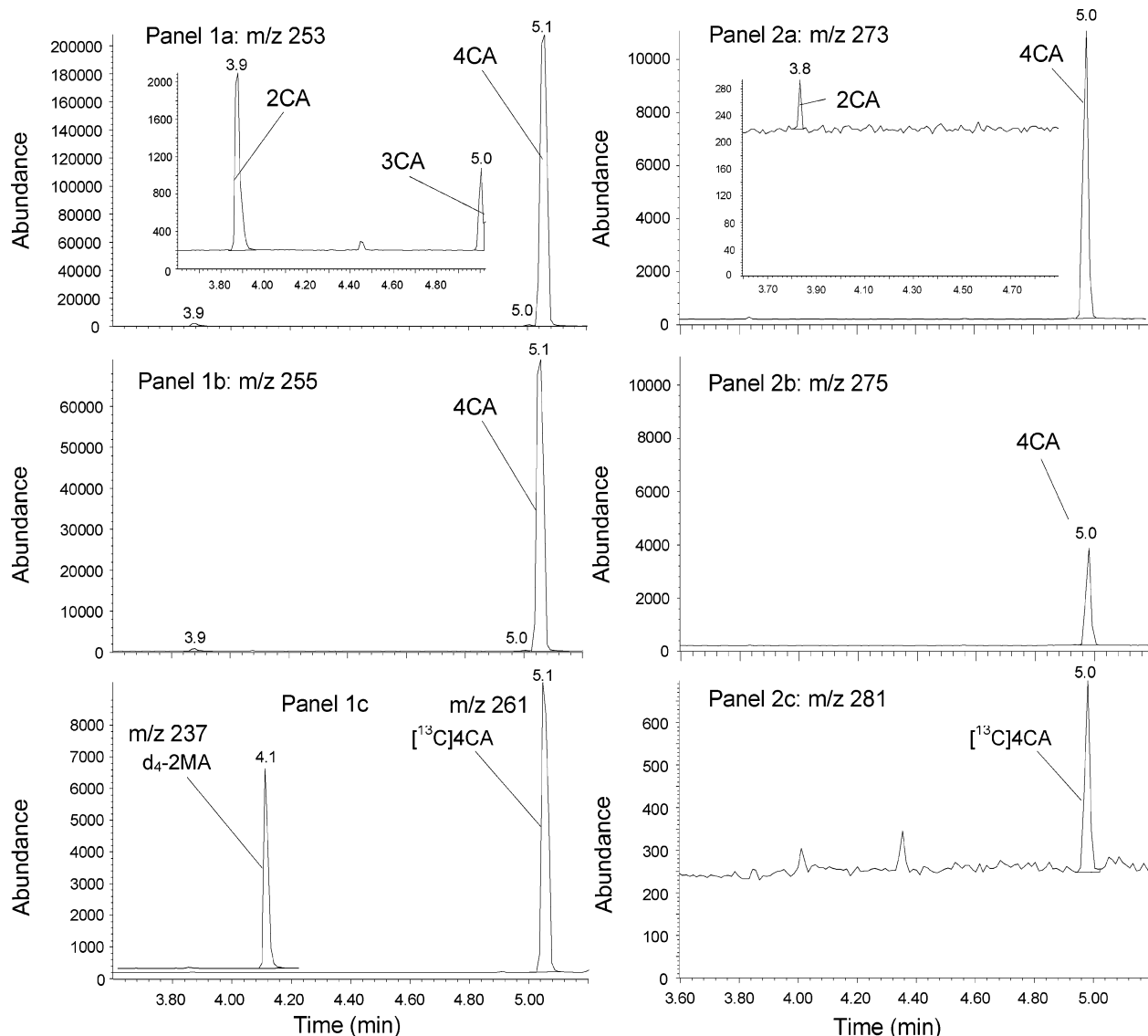


FIGURE 2. Mass chromatograms of extracted Hb from a CNB-exposed worker. The arylamines were separated by GC prior to mass analysis. Panel 1: Selected ions were monitored in NCI mode. Single-ion chromatograms of m/z 253 (panel 1a) and 255 (panel 1b) were acquired and corresponded to $[M - HF]^-$ ions of the PFPA-derivatized amines 2CA, 3CA, and 4CA. The ions m/z 261 and 237 (panel 1c) were acquired for quantitation of the internal standards $[^{13}C]4CA$ and d_4 -2MA, respectively. Both 2CA and 4CA were quantified from their ion abundance at m/z 253. Panel 2: Selected ions were monitored in EI mode. Single-ion chromatograms of m/z 273 (panel 2a) and 275 (Panel 2b) were acquired which corresponded to $[M]^+$ ions of the PFPA-derivatized amines 2CA, 3CA, and 4CA. The ion m/z 281 (panel 2c) was acquired for quantitation of the internal standard $[^{13}C]4CA$.

which corresponded to the natural isotopic abundance of one chlorine atom present in a molecule. Single-ion chromatograms acquired in NCI mode are presented in Figure 2. The presented chromatograms were from hexane extracts of the Hb hydrolysates from an exposed worker. The ions m/z 253 and 255 are presented with internal standards $[^{13}C]$ -4CA, m/z 261, and d_4 -2MA, m/z 237. The major peak in the single-ion chromatogram eluted at 5.1 min and corresponded to 4CA. There were two additional peaks at 3.9 and 5.0 min, which corresponded to 2CA and 3CA, respectively. The inset in Figure 2 showed the single-ion chromatogram m/z 253 peak expanded between 3.6 and 5 min. Baseline resolution of the 3CA peak from the shoulder of 4CA peak was not achieved in all cases under the described chromatographic conditions. Additionally, the area of the 3CA peak was more than 100-fold lower than that of the 4CA peak. Therefore, the determination of 3CA did not match the quality criteria described for 2CA and 4CA.

2CA was detected in 38 of 39 exposed Hb samples, 3CA was detected in 32 of 39 exposed Hb samples, and 4CA was

detected in all the exposed Hb samples within the determination limit of the assay (0.38 pg/mg of Hb) in NCI mode. 2CA was detected in 13 of 15 factory control Hb samples, 3CA was detected in 8 of 15 factory control Hb samples, and 4CA was detected in all of the factory control Hb samples. However, there were no 2CA, 3CA, or 4CA Hb adducts detected in the nonfactory control samples, except for one sample, which was positive for 4CA.

The level of hydrolyzable Hb-arylamine adducts present in each Hb was calculated from a calibration line of known standards spiked into bovine Hb and taken through the assay procedure. The levels of hydrolyzable adducts of 2CA and 4CA (pg/mg of Hb) determined in CNB workers are presented in Table 2. 4CA-Hb adduct levels ranged over 20-fold in exposed workers. The levels of 2CA-Hb adducts were approximately 11-fold lower than the 4CA-Hb adduct levels. The levels of 3CA were in the same range as for the 2CA-Hb adducts and were present at a median level (25th, 75th percentile) of 12.7 pg/mg of Hb (5.74, 44.0) in the exposed workers and 2.9 pg/mg of Hb (0, 9.2) in the controls.

TABLE 2. Hb Adduct Levels (pg/mg) of 2CNB and 4CNB Found in Exposed Workers and in the Factory Controls^a

	mean	median (25th, 75th, 90 th percentile)
Exposed Workers		
2CA	92.4	82.9 (56.6, 143, 166)
4CA	1037	1013 (750, 1456, 1834)
Factory Controls		
2CA	21.4	5.3 (1.8, 38.3, 80.8)
4CA	217	35.9 (17.3, 483, 793)

^a These data were obtained with GC-MS in NCI mode. The adduct levels were significantly lower in the controls (Mann-Whitney test, $p < 0.01$).

Other possible products such as 2,6-dichloroaniline (m/z 271) and 2,4-dichloroaniline (m/z 287) were not found in these workers.

The levels of the adducts were also determined by GC-MS with EI ionization (Figure 2). In EI mode the abundance

of ion fragments $[M]^+$ at m/z 273 and 275 was acquired for characterization of 2CA, 3CA, and 4CA. These fragments were observed at an abundance ratio of 3:1, which corresponded to the natural isotopic abundance of one chlorine atom present in a molecule. Additional ions were acquired for further product characterization (Table 1, Figure 3).

Correlation between Hb Adduct Levels. The levels of 2CA and 4CA (pg/mg of Hb) determined in the workers by GC-MS-EI were a very close approximation to the levels determined by GC-MS-NCI. The correlation (Pearson correlation method) between 4CA levels determined by GC-MS-NCI and GC-MS-EI was 0.99 ($p < 0.01$) (Figure 4). The correlation between 2CA levels determined by GC-MS-NCI and GC-MS-EI was 0.96 ($p < 0.01$). The correlation between 2CA and 4CA levels, determined by GC-MS-NCI, was 0.87 ($p < 0.01$) and confirmed that there was a strong association between Hb adduct levels of 2CA and 4CA in these workers.

Comparison of Air Levels with Hemoglobin Adduct Levels. The 8 h time-weighted average (TWA) exposure levels of 2CNB, 3CNB, and 4CNB were determined in a subset of

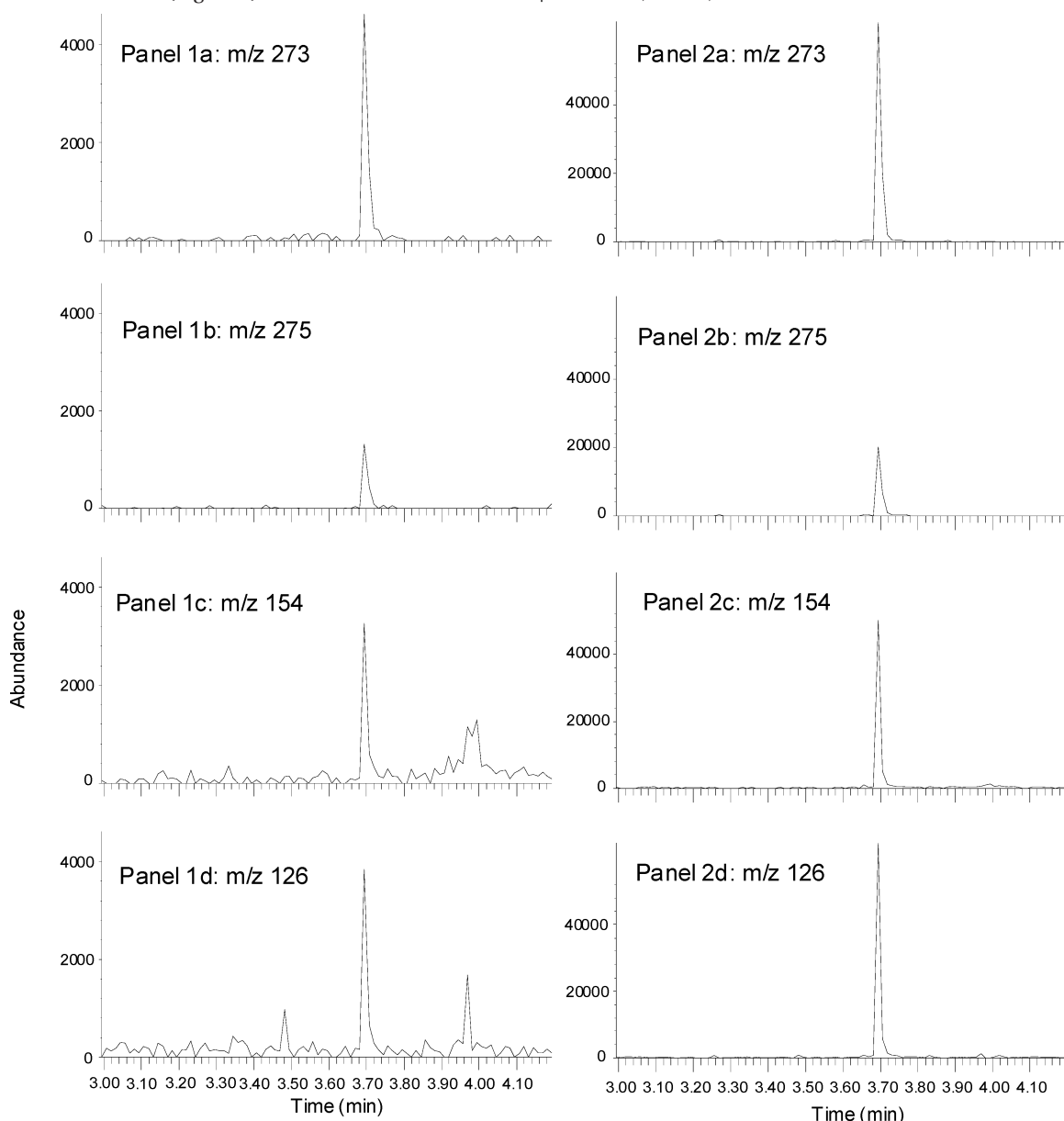


FIGURE 3. Full-scan mass spectra of PFPA-derivatized 4CA taken in EI mode. The amine 4CA was separated on a fused silica capillary column (Rtx-5MS (Restek), 15 m \times 0.25 mm, 0.25 μ m film thickness) and a full spectrum was, m/z 50–350, acquired. Panels 1a–d and 2a–d: Single-ion chromatograms of m/z 273, 275, 154, and 126, which correspond to the most abundant fragments formed by EI ionization of 4CA determined in an exposed worker (panels 1a–d) and the 4CA authentic standard (panels 2a–d).

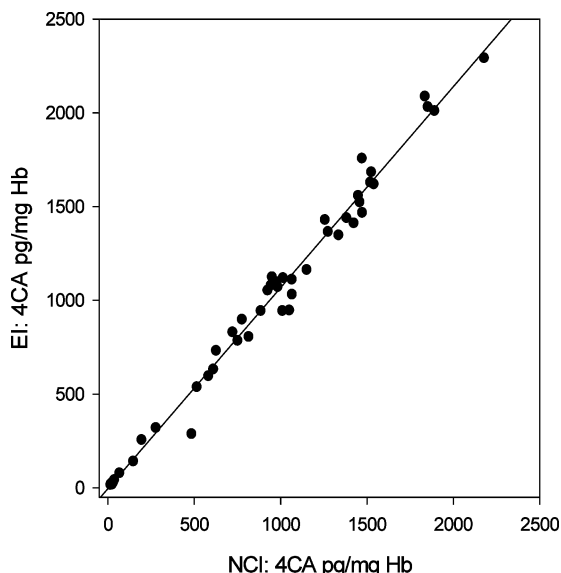


FIGURE 4. Correlation ($r = 0.99$) of Hb adducts of 4CNB quantified with two methods, GC-MS (i) with NCI and (ii) with EI detection.

exposed workers ($n = 19$) using personal air samplers strapped to each worker during the course of their work shift (NIOSH method 2005). 2CNB and 4CNB were found in all samplers. In contrast, 3CNB was found only in three samplers. The median (25th, 75th, 90th percentile, mean) 8 h TWA exposure levels for 2CNB and 4CNB were 0.37 mg/m^3 (0.23, 0.73, 0.96, 0.49) and 0.87 mg/m^3 (0.63, 1.2, 3.38, 1.17), respectively. The air levels of 2CNB and 4CNB correlate with $r = 0.82$ ($p < 0.01$). The median levels of 2CNB and 4CNB were below the OSHA permissible exposure level (PEL) and the U.K. exposure limits set at 1 mg/m^3 . The air levels of 2CA and 4CA did not correlate with the Hb adduct levels of 2CA and 4CA ($r < 0.2$, $p > 0.05$), respectively. This may indicate that other routes of exposure are important.

Both the air level data and the Hb adduct data were determined in 19 workers. The median (25th, 75th, 90th percentile, mean) Hb adduct levels for 2CA and 4CA were $0.0972 \text{ } \mu\text{g/g}$ of Hb (0.064, 0.158, 0.200, 0.106) and $1.254 \text{ } \mu\text{g/g}$ of Hb (0.814, 1.456, 1.517, 1.076), respectively. The Hb adduct levels of 2CA and 4CA of the 19 workers correlate with $r = 0.86$ ($p < 0.01$). The median Hb adduct levels of 4CA were 13 times larger than the median Hb adduct levels of 2CA. The median air levels of 4CA were ca. 2.4 times larger than the median air levels of 2CA.

The inhaled exposure dose of 2CNB and 4CNB was estimated from the air measurements assuming that a 70 kg worker inhales 9.6 m^3 of air during an average 8 h work shift. Thus, the median dose per worker was 3.54 mg (2.23, 7.02, 9.17) for 2CNB and 8.38 mg (6.05, 11.5, 32.4) for 4CNB. These values were compared to an estimated absorbed dose calculated from the quantified Hb adduct levels. It was assumed that the same percentage of the dose binds in rats and humans and that in humans the steady-state Hb adduct level is 60 times greater than after a single dose (21, 29, 30, 31). The percentage of the dose bound in rat was 0.03% for 2CNB and 3.12% for 4CNB (22). Therefore, this gave an estimated median absorbed dose of 5.53 mg (3.63, 9.00, 11.4) and 0.70 mg (0.45, 0.81, 0.84) for 2CNB and 4CNB, respectively. The median levels of the estimated absorbed dose from the Hb adduct calculations were 1.6 times higher for 2CNB and 12 times lower for 4CNB than the median levels of the inhaled dose. This divergence may be explained by the fact that 2CNB has a higher vapor pressure at room temperature than 4CNB. Alternatively, since the other physicochemical properties were similar, it is possible that

other routes of exposure are important. Another possibility is that in humans 4CNB binds to Hb to a lesser extent than in rats. For 2CNB it appears that similar amounts bind to Hb in rats and humans. Furthermore, Hb adduct levels reflect all routes of exposure; they integrate the exposure over the lifetime of the erythrocytes, as well as take into account individual variation in uptake, metabolism, and absorbance. These factors are not reflected in air measurements.

Chloronitrobenzene Exposure and Cancer Risk. An excess lifetime cancer risk (ELCR) for exposure to these chemicals was estimated using the following formula (32, 33; <http://risk.lsd.ornl.gov>): $\text{ELCR} = \text{cancer slope factor} \times \text{human dose}$. The cancer slope factors for 2CNB (7, 9) and 4CNB are $9.7\text{E}-03$ and $6.7\text{E}-03$ ($(\text{mg/kg})/\text{day}$) $^{-1}$, respectively. To compare risk assessment from air and Hb adduct levels, the data for which both values were available were used (see above). The median doses for 19 exposed workers, estimated from the Hb adduct levels of 2CNB and 4CNB, were 79.9 and $9.9 \text{ } \mu\text{g/kg}$. The workers were not exposed 7 days per week, 52 weeks per year. For the calculation with air levels the daily dose has to be corrected with the factor 5 days/7 days \times 50 weeks/52 weeks. For Hb adducts, days without exposure are included. Therefore, the dose deduced from Hb adducts was not corrected for the days without exposure. Since workers are not exposed for a lifetime of 70 years to the occupational pollutants, but for 40 years of work life, the dose was corrected with the factor 40/70. This gave ELCR values for exposure to 2CNB and 4CNB of 4.4 in 10000 and 3.8 in 100000, respectively. To directly compare the values with those of workers exposed to dinitrotoluenes (1.3 in 100 (29)) and 2,4,6-trinitrotoluene (8.8 in 10000 (31)), the ELCR for the 95th percentile level of all 39 exposed workers was estimated: 9.0 in 10000 for 2CNB and 5.7 in 100000 for 4CNB. A risk of 1 in 10^6 is perceived as a virtually safe dose. Given these ELCR values exceed the safe dose, there is an increased risk from exposure, especially resulting from 2CNB exposure. Taking the median air levels would yield a 1.6-fold smaller risk for 2CNB but a 12-fold higher risk for 4CNB. Therefore, for 4CNB the Hb adduct levels might underestimate the risk.

Another way of estimating this human cancer risk is to use the human exposure rodent potency (HERP) index (34), which is calculated using the daily dose rate administered to rodents that results in a 50% increased likelihood of tumor development (TD_{50}) (35, <http://potency.berkeley.edu/>). The HERP index corresponds to a ratio of the daily human exposure and the rodent TD_{50} value expressed as a percentage. The ratio between the median dose deduced from Hb adducts (corrected with the factor 40/70; see above) for 2CNB and 4CNB and the TD_{50} value determined in mice (157 (mg/kg)/day 2CNB, 473 (mg/kg)/day 4CNB) is 0.029% and 0.0012%, respectively. This is equal circa to a risk (HERP of 0.00001% approximately = 1 in 10^6 , (34)) of 2.9 in 1000 and 1.2 in 10000 for 2CNB and 4CNB, respectively. Strictly speaking, a comparison should be made to the value in rats for both cancer slope factor and TD_{50} , since the human Hb adduct levels were transformed into an absorbed dose ((mg/kg)/day) on the basis of the Hb binding of 2CNB and 4CNB in rat. However, both the cancer slope factor and TD_{50} are based on the mouse because the rat results were not considered positive for 2CNB and 4CNB. The risk calculated from the HERP index was higher than the risk deduced from the method by the EPA by a factor of 6.6 for 2CNB and 3.2 for 4CNB. The difference is due to the use of time-to-tumor data in the estimation of TD_{50} , whereas EPA used summary data only. The time-to-tumor results are more potent because they adjust for differences in toxicity among dose groups and for differences in the time pattern of tumor incidence. Additionally, for 2CNB both male and female mouse results were used in the HERP calculation but only females in the EPA calculation.

TABLE 3. Nonspecific Health Effects and Clinical Blood and Urine Parameters Observed in Workers Exposed to Chloronitrobenzenes and Factory Controls

	comparison of health params between exposed and control workers			comparison of health params between exposed and control workers	
	exposed	control		exposed	control
fatigue	6 ^a	0	urine rbc's	4 ^a	0
headache	3 ^a	0	urine wbc's	2 ^a	0
dizziness	6 ^a	2	urine protein	3 ^a	3
insomnia	4 ^a	2	urine bilirubin	2 ^a	0
eye irritation	4 ^a	0	urine glucose	2 ^a	1
skin irritation	1 ^a	0	metHb level $\geq 2\%$	8 ^a	3
dyspnea	3 ^a	1	urine pH	5.5 \pm 0.5 ^{b,c}	5.6 \pm 0.5
splenomegaly	7 ^a	0	Hb concn ^d (g/L)	140 \pm 10 ^{b,e}	148 \pm 9
hepatomegaly	1 ^a	0	rbc count ^d ($\times 10^{12}/L$)	4.3 \pm 0.5 ^{b,e}	4.8 \pm 0.4
ECG-1	5 ^a	1	wbc count ^d ($\times 10^9/L$)	6.3 \pm 2.1 ^{b,e}	7.8 \pm 2.2
ECG-2	3 ^a	0	MetHb level ^d (%)	1.3 \pm 0.7 ^{b,e}	1.6 \pm 0.5
ECG-3	5 ^a	0	sgpt level ^d (U/L)	19.5 \pm 6.9 ^{b,c}	19.8 \pm 7.9

^a Comparison of exposed and control workers using the Fisher exact test ($p > 0.05$). ^b Comparison of exposed and control workers using the Mann–Whitney test. ^c $p > 0.05$. ^d Comparison only among male workers. ^e $p < 0.05$.

Since the air levels were close to the PELs set by OSHA, additional cancer cases may arise in workers exposed to CNB in Europe and the United States. For workers exposed to the PEL of 2CNB and 4CNB, a total ELCR of 8.8 in 10000 was estimated. Gold et al. (36) showed also for other occupational pollutants that the PEL values appear to be too high when compared with the cancer risk deduced from the animal experiments.

Chloronitrobenzene Exposure and Health Effects. A full medical examination was performed on each worker. The results are summarized in Table 3. Biological effects such as fatigue, headache, eye irritation, skin irritation, hepatomegaly, splenomegaly, and cardiovascular effects (ECG2, ECG3) were found only in exposed workers. Dizziness, insomnia, dyspnea, and a cardiovascular effect (ECG1) were found in exposed workers and controls. The differences between exposed workers and controls were statistically not significant (Fisher's exact test). There were no apparent increases in spleen sizes in any of the 17 factory control workers, but 7 out of 40 exposed workers showed an increase in relative spleen sizes. Toxicological effects in the spleen have been associated with exposure to arylamines and nitroarenes (37–39). With respect to the clinical urine parameters, rbc's, wbc's, and bilirubin were present only in the exposed workers. The numbers were too small to reach statistical significance. Most blood parameters were significantly different between the two groups. The median rbc and wbc counts, Hb concentration, and metHb level were significantly lower in the exposed male workers than in the male controls. The actual levels of metHb in control and exposed workers were less than 2%, which suggested that no real effect with respect to elevated metHb had occurred. In age-adjusted (two age groups) or in work-year-adjusted (two work year groups) groups, the relative median levels of the blood parameters did not change between the controls and exposed workers. Therefore, the decrease of the blood parameter levels appears to be exposure related.

The health effects were compared with the Hb adduct levels, age, work years, and number of packs of cigarettes smoked per day using the Mann–Whitney test. The median Hb adduct levels were higher in workers with fatigue, eye irritation, splenomegaly, ECG1, ECG2, and ECG3 ($p > 0.05$). This corresponds to effects documented in the literature for 4CNB-exposed workers (Hazardous Substances Data Bank, <http://toxnet.nlm.nih.gov>). With the exception of protein found in urine, all other negative urinary markers were present at higher median Hb adduct levels ($p > 0.05$). This might be explained with the toxic properties of CNB on the kidneys as reported in animals (7, 9). The continuous clinical

blood and urine parameters were compared between the first and last tertiles of the categorized Hb adduct levels using the Mann–Whitney test. The urine pH, wbc count, and sgpt level were lower at higher adduct levels. The decrease of the wbc count could be a consequence of the immunotoxic properties of CNB (7–9). The Hb concentration and rbc count were lower at higher Hb adduct levels, indicating damage to the erythrocytes by CNB. This confirms the effects seen in animals (7–9). The median adduct levels were higher in the cases with metHb level $> 2\%$ compared to the cases with metHb level $< 2\%$. However, this relationship was not statistically significant. Therefore, it appeared that metHb formation was associated more with acute exposure to high levels of 4CNB than with chronic exposure. It may be concluded that in chronically exposed workers the 4CA–Hb adduct levels were not sufficiently high to induce metHb formation, or that following chronic exposure the body adapted and enzyme systems responsible for removal of metHb were induced and effectively prevented the buildup of metHb (40). Indeed, it is known that metHb formation is an endogenous byproduct of normal physiological respiratory processes (40), with low levels (0.8–2%) being found continuously in vivo. The unspecific health effects were compared to the work years and the number of packs of cigarettes smoked per day using the Mann–Whitney test. The people suffering from dizziness and dyspnea had significantly higher ($p < 0.05$) cigarette consumption. Protein in urine was more prevalent in workers who had worked more years ($p < 0.05$). For age, the wbc levels were significantly higher ($p < 0.05$) in older people. People with eye irritation had significantly less work years. Therefore, cases of dizziness, dyspnea, and urine protein found in controls relate to smoking, smoking, and work years, respectively.

Hb adducts provided a good estimate of chronic exposure and of body burden for the reactive metabolites of CNB, namely, the nitrosoarenes and *N*-hydroxyarylamines. Hb adducts showed a dose-related effect with nonspecific symptoms, indicative of adverse health effects in these chronically exposed workers. However, the number of investigated workers was too small to obtain statistically significant results. At present, we are unable to calculate odd ratios for biological effects resulting from CNB exposure, as demonstrated for workers exposed to dinitrotoluenes (29) and 2,4,6-trinitrotoluene (31). However, Hb adducts did provide a chronic marker of the internal biologically effective dose of electrophiles known to be involved in the toxification processes of 4CNB.

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