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Urinary metabolites and health effects in workers exposed chronically to chloronitrobenzene

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Abstract

For workers exposed to 4-chloronitrobenzene (4CNB), the major metabolites were determined. Urine were analysed before and after acid hydrolysis to qualify the free and conjugated metabolites of 4CNB. Three conjugated metabolites were identified in exposed workers: the mercapturic acid *N*-acetyl-*S*-(4-nitrophenyl)-L-cysteine (NANPC) was the only metabolite detected in non-hydrolysed urine, and accounted for approximately 51% of the total metabolites detected. The two remaining metabolites 4-chloroaniline (4CA) and 2-chloro-5-nitrophenol (CNP) were identified as cleavage products in hydrolysed urine, and accounted for approximately 18 and 30% of the total metabolites detected, respectively. No metabolites were found in factory controls within the limits of quantitation (LOQ) of the assay. There is a moderate correlation between NANPC and both 4CA and CNP. The correlation between 4CA and CNP is minor. The correlation between the total metabolites and both 4CA and CNP are good. The best correlation was found between the total metabolites and NANPC. There is a moderate inverse correlation between age and the creatinine levels. The raw metabolite levels CNP and NANPC decrease with age. The urine metabolites increase and correlate significantly with the creatinine levels. 4CA, NANPC and the total metabolite levels correlate with the haemoglobin adduct levels. NANPC is the most appropriate biomarker in the urine for a recent absorbed dose of 4CNB, since NANPC reflects the levels of 4CA and CNP and is the most prevalent metabolite detected in all the exposed workers.

Keywords: *4-chloronitrobenzene, biomonitoring, urinary metabolites, 4-chloroaniline, haemoglobin adducts*

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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 (NTP 1993, Gdch 1992a,b). Although these chemicals are solids at room temperature, the vapour pressures of these chemicals are sufficiently high to result in a significant

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exposure via inhalation (NIOSH/OSHA 1978). 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 been reported in samples taken in the environment (Feltes et al. 1990). The US Environmental Protection Agency (EPA) lists 4CNB as a possible human carcinogen based on no evidence in humans and positive evidence in mice (US EPA 2002a). 2CNB induced tumours in different organs of rats and in the liver of mice (US EPA 2002b). 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^{-1} day^{-1}) for 2CNB and 4CNB. No carcinogenicity studies have been performed for 3CNB. 2CNB and 4CNB were mutagenic in *Salmonella typhimurium* with S9 activation (Mortelsman & Zeiger 2000). 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 (NTP 1993). In male F344 or Sprague–Dawley rats given a single oral radiolabelled 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, respectively. 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 et al. (1993) and Yoshida (1994) 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 dermal routes (Yoshida et al. 1993). The main metabolites were *N*-acetyl-*S*-(4-nitrophenyl)-L-crysteine (NANPC) (48% of the total metabolites), 4-chloroaniline (29.9%) (4CA), 2-chloro-5-nitrophenol (CNP) (12.2%), and 2-amino-5-chlorophenol (8.7%). To date there are no publications about the urinary excretory products of 4CNB in chronically exposed workers. Therefore, we have devised a method for the identification and quantitation of the same urinary metabolites of 4CNB, outlined above, to biomonitor a group of Chinese workers chronically exposed to 4CNB. The urinary metabolite levels were compared with the air levels and to the health effects.

Materials and methods

2,4-Dichloroaniline (99%) (24DCA), concentrated hydrochloric acid 37% (concentrated HCl), 4-chloroacetanilide (97%) (4CAA), 4-chloroaniline (99%) (4CA), 4-chloronitrobenzene (99%) (4CNB), 4-nitrophenol (99%) (4NP), 4-nitrophenol-glucuronide (4NP-Gluc), and 4-nitrophenol sulfate (4NP-sulfate) were obtained from Aldrich (St. Louis, MI, USA). Ammonium dihydrogen orthophosphate ($NH_4H_2PO_4$) and sodium hydroxide (98%) (NaOH) were obtained from BDH Chemical Ltd. (Poole, UK). HPLC-grade *ortho*-phosphoric acid, acetonitrile (AcCN) and methanol (MeOH) were obtained from Fisher Scientific (Pittsburgh, PA, USA). l-Octanesulfonic acid sodium salt (99%) was obtained from Fluka (Buchs, Switzerland). *N*-acetyl-*S*-(4-nitrophenyl)-L-cysteine (>98%) (NANPC) was obtained from TCI Tokyo Kasei Organic Chemicals (Tokyo, Japan). 2-Chloro-5-nitrophenol (CNP), 2-acetylamino-5-chlorophenol (AACP), 4-chloro-oxanilic acid (4COA), and 4-chloroaniline-N-glucuronide (4CA-N-gluc) were synthesized as described below.

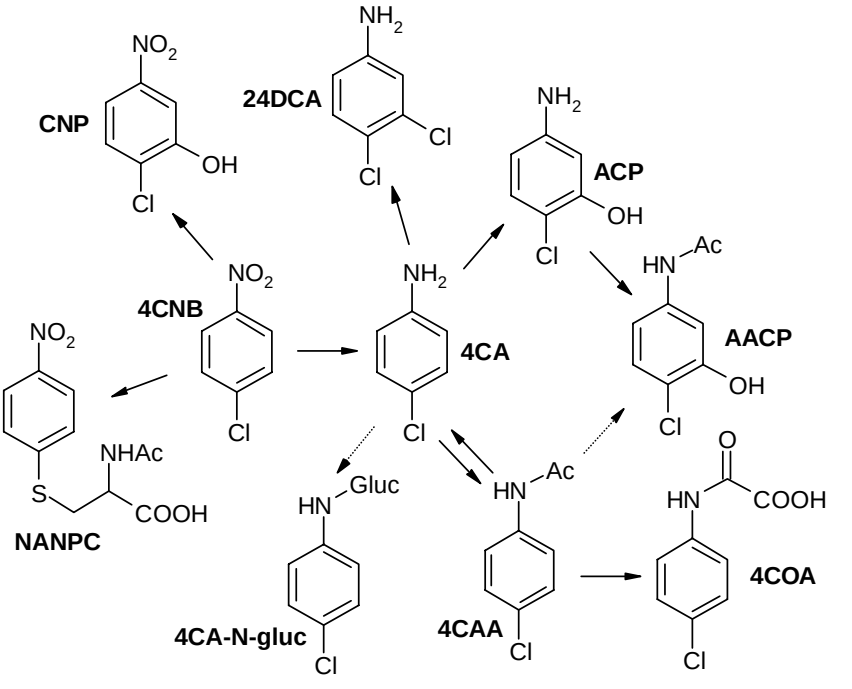

Figure 1. Metabolic pathway of 4CNB in rats and acutely poisoned humans. The scheme was adapted from Yoshida et al. (1993). The metabolism of 4CNB occurs through three distinct pathways, replacement of the chloro group with glutathione to yield the mercapturic acid, ring hydroxylation and phase II conjugation, or nitro reduction. Following nitro reduction, 4CA is further metabolized through the amino group to a N-conjugate or ring-hydroxylated or chlorinated. The full lines represent pathways where the products have been identified, and dashed arrows represent postulated pathways. CNP, 2-chloro-5-nitrophenol; 4CA, 4-chloroaniline; 24DCA, 2,4-dichloroaniline; ACP, 2-amino-5-chlorophenol; 4CAA, 4-chloroacetanilide; AACP, 2-acetylamino-5-chlorophenol; 4CA-N-gluc, 4-chloroaniline-N-glucuronide; and 4COA, 4-chloro-oxanilic acid.

^{1}H-NMR was performed on a Bruker NMR-200-MHz machine. The compounds were in $(CD_3)_2SO$ with TMS as a reference.

CNP was synthesized according to Mendola & Wray (1894). MS: *m/z* 173 $[M]^+$ and 127 $[M - NO_2]^+$. 500 MHz ^{1}H-NMR δ: 7.74 (d, $\mathcal{J}=2.5$ Hz, H-6), 7.67 (dd, $\mathcal{J}=8.8$ Hz, $\mathcal{J}=2.4$ Hz, H-4), 7.63 (d, $\mathcal{J}=8.7$ Hz, H-3), 3.5 (br s, OH).

AACP was synthesized according to Katz & Cohen (1953) starting from 2-amino-nitrophenol through the following intermediates: 2-acetylamino-5-nitrophenyl acetate: MS: *m/z* 238 $[M]^+$, 196 [M–ketene]$^+$ and 154 [M–ketene]$^+$. ^{1}H-NMR δ: 8.5 (dd, $\mathcal{J}=9.0$ Hz, $\mathcal{J}=1.83$ Hz, H-4), 8.25 (d, $\mathcal{J}=1.83$ Hz, H-6), 8.19 (d, $\mathcal{J}=7.8$ Hz, H-3), 2.4 (s, OAc), 2.27 (s, NAc). N1-(2-hydroxy-4-nitrophenyl) acetamide: MS: *m/z*: 166 $[M]^+$ and 124 [M–ketene]$^+$. ^{1}H-NMR δ: 8.4 (d, $\mathcal{J}=8.8$ Hz, H-5), 7.84 (d, $\mathcal{J}=2.6$ Hz, H-3), 7.80 (d, $\mathcal{J}=8.8$ Hz, H-6), 2.26 (s, NAc). N1-(4-amino-2-hydroxyphenyl) acetamide: MS: *m/z* 166 $[M]^+$ and 124 [M–ketene]$^+$. ^{1}H-NMR δ: 7.1 (d, $\mathcal{J}=8.4$ Hz, H-6), 6.18 (d, $\mathcal{J}=2.27$ Hz, H-3), 6.08 (dd, $\mathcal{J}=8.4$ Hz, $\mathcal{J}=2.4$ Hz, H-5), 4.92 (br s, NH_2), 2.09 (s, NAc).

AACP: MS: *m/z* 185 $[M]^+$ and 143 [M–ketene]$^+$. ^{1}H-NMR δ: 10.4 (s, NH), 9.4 (br s, OH), 7.9 (dd, $\mathcal{J}=8.5$ Hz, $\mathcal{J}=2.2$ Hz, H-4), 6.97 (d, $\mathcal{J}=2.2$ Hz, H-6), 6.89 (d, $\mathcal{J}=8.4$ Hz, H-3), 2.17 (s, NAc).

4COA. The potential phase II conjugate of 4CA, 4COA, was synthesized according to the procedure of Kiese & Lenk (1969). MS: m/z 199 $[M]^+$, 154 $[M-CO_2H]^+$, 126 $[M-COCO_2H]^+$ and 111 $[M-COCO_2NH]^+$, 500 MHz ^{1}H-NMR δ: 11.02 (s, CO_2H), 10.85 (s, NH), 7.8 (dd, $J=8.9$ Hz, $J=2.6$ Hz, H-2,6), 7.4 (dd, $J=8.9$ Hz, $J=2.6$ Hz, H-3, 5).

4-Chloroaniline-N-glucuronide (4CA-N-gluc). The possible phase II conjugate of 4-chloroaniline (4CA), 4CA-N-gluc, was synthesized according to procedure of Lilienblum & Bock (1984). 500 MHz ^{1}H-NMR δ: 7.2 (d, $J=8.7$ Hz, H-2,6), 6.7 (d, $J=8.8$ Hz, H-3,5), 6.5 (1H, d, $J=7.9$ Hz, cyclonyl H-12), 5.01 (m, H-9, H-10, H-11), 4.42 (m, 8-H).

Questionnaire and medical examination

The CNB study was carried out in Tainjing Chemical Factory (in Tainjing city). The study was performed in accordance with the principles embodied in the Declaration of Helsinki (http://www.wma.net/e/policy/b3.htm). Informed consent was obtained from each worker. Urine collection, medical examination and filling out the questionnaire were all performed in the same week. Participants were interviewed with a questionnaire about their general status, exposure history, smoking habits, previous medical record and present symptoms. For the present study of urinary metabolites 38 (36 males) exposed workers and ten (nine males) non-exposed workers were recruited. The number of smokers in the exposed and control worker group were 26 and six, respectively. The median age ranges of the exposed workers and control workers were 32.0 (18.0–49.4) and 43.5 (24.0–47.0) years, respectively. The median number of work years in the present factory were 6 (1–16) and 11 (3–19) years in exposed and control workers, respectively. The medical department of the Chinese Academy of Preventive Medicine performed the following examinations: (1) physical examinations: blood pressure, cardiovascular system, nervous system, and heart rate; (2) routine blood (red blood cell (rbc) count, white blood cell (wbc) count, methaemoglobin (metHb), serum glutamic pyruvic transaminase (sgpt), and urine tests (pH, bilirubin, protein, wbc, rbc, glucose); (3) electrocardiogram (ECG) (ECG1, sinus tachycardia, sinus bradycardia; ECG2, arrhythmia; ECG3, conduction); (4) ultrasonic type B examination for liver and spleen; and (5) serological assays of hepatitis B antigens and antibodies were conducted, because hepatitis B is rather common in China, and liver damage can also be caused by some of the nitroarenes.

Collection of urine samples

Spot urine samples were collected in 50-ml polypropylene tubes. An additional eight samples were collected from non-exposed volunteers from the Department of Environmental and Occupational Medicine, Newcastle upon Tyne. All urine samples were stored at -20°C immediately after collection. Urine samples were analysed in duplicate before and after acid hydrolysis to determine both conjugated and non-conjugated metabolites, respectively. In non-hydrolysed urine 4CNB and the metabolites NANPC, 4CA-N-gluc, 4COA, 4CA, AACP, CNP, 4CAA, and 24DCA were screened for. In hydrolysed urine 4CNB and the metabolites 4CA, CNP and 24DCA were screened for.

Method A: Determination of metabolites in acid-hydrolysed urine

The urine samples were thawed at room temperature and vortex mixed for 1 min. For each sample, an aliquot (2 ml) was transferred to 10-ml polypropylene screw capped tube (Corning, Acton, MA, USA; 100 × 22 mm) with teflon liner (Supelco, Bellefonte, PA, USA). The samples were spiked with 20 µl 4NP internal standard (100 µmol ml^{-1}). Following addition of 0.1 vols of concentrated HCl, the samples were incubated at 100°C (Techne, Driblock D8-3 column heater, Thistle Scientific Ltd, Glasgow, UK) for 20 h. The acidified samples were neutralized with 5.0 M NaOH then centrifuged 2000*g* for 15 min. An aliquot of each sample (500 µl) was transferred to an HPLC vial (32 × 12 mm) for automated analysis.

Separation and detection of the cleaved conjugates was performed using high-performance liquid chromatography (HPLC) coupled with ultra-violet (UV) detection. The HPLC system consisted of a Kontron Instrument 460 autosampler, 420 pump, 425 gradient former, 430 UV detector and a Jones Chromatography HPLC 7955 column chiller (#7955, Jones Chromatography Ltd., Hengoed, UK). All mobile phases (MP) were purged with helium to displace dissolved oxygen. Each sample (40 µl) was injected onto a LiChrospher RP-Select B column (125 × 4 mm i.d., 5 µm; Merk, Darmstadt, Germany) with pre-column (4 × 4 mm). The metabolites (4CA, CNP, 4CNB, 24DCA) and internal standard (4NP) were eluted under gradient conditions (0–20 min, 100:0 MP1:MP2; 21–35 min, 100:0 to 70:30 MP1:MP2; 36–40 min, 70:30 MP1:MP2, 41–50 min 70:30 to 100:0 MP1:MP2, 51–59 min 100:0 MP1:MP2) at 1 ml min^{-1}, 30°C with a detection wavelength of 240 nm. The mobile phases consisted of (MP1) 80:20 v/v (1.2 mM 1-octanesulfonic acid sodium salt, 5 mM $NH_4H_2PO_4$ buffer pH 3.6): MeOH and (MP2) 40:60 v/v (1.2 mM 1-octanesulfonic acid sodium salt, 5 mM $NH_4H_2PO_4$ buffer pH 3.6): MeOH.

Optimization of hydrolysis conditions using 4NP-glucuronide and 4NP-sulfate as model substrates

Aliquots (2 ml) of 4NP glucuronide or sulfate conjugate solutions (40 µmol l^{-1}) were pipetted into screw capped tubes with Teflon liners and spiked with 10 µl of 4CA, (1 mmol l^{-1}) used as internal standard. Concentrated HCl (0.1 vols) was added, and the samples were incubated at 100°C for the designated time point. The time points for hydrolysis of 4NP-gluc were 0, 45, 60, 120, 240, 1200 and 1440 min, and for 4NP-sulfate were 0, 15, 30, 60 and 120 min. After hydrolysis the samples were neutralized with 5.0 M NaOH, then aliquots (20 µl) were injected onto an RP-Select B column under the conditions described in method A. The peak areas of 4NP released with respect to 4CA were plotted against time. The plot of 4NP released from 4NP-sulfate indicated that by 15 min (data not shown), under the described assay conditions, complete hydrolysis had occurred. The plot of 4NP released from 4NP-glucuronide indicated that a longer time period was required for total cleavage of the conjugate (1200 min, 20 h). Longer hydrolysis time (>20 h) gave lower yields.

Hydrolysis of 4CA-N-gluc and 4COA

The metabolites 4CA-N-gluc and 4COA were synthesized as potential phase II conjugates of 4CA. The hydrolysis conditions required to cleave these potential phase II conjugates and release 4CA were investigated to determine if they gave similar

profiles to those required to cleave the unknown 4CA conjugates in the urine samples. Aliquots (2 ml) of aqueous solutions of 4CA-N-gluc and 4COA (40 μmol l^{-1}) were pipetted into 10-ml screw capped polypropylene tubes with Teflon liners, and spiked with 10 μl of 4NP (1 mmol l^{-1}), used as internal standard. Concentrated HCl (0.1 vols) was added and the samples were incubated at 100°C for the designated time point. The time points for hydrolysis of 4CA-N-gluc and 4COA were 0, 5, 15, 30, 60, 120 and 240 min. After incubation the samples were neutralized with 5.0 M NaOH, then aliquots (20 μl) were injected onto a RP Select B column and analysed according to the described conditions in method A.

Determination of metabolites in non-hydrolysed urine: Method B: determination of all metabolites except NANPC

A chromatographic system with coupled UV detection was devised to analyse all the potential urinary metabolites of 4CNB, except for NANPC. The urine samples were thawed at room temperature and vortex mixed for 1 min. An aliquot (2 ml) from each sample was transferred to a 10-ml polypropylene screw capped tube with a Teflon liner. The sample was spiked with 20 μl 4NP (100 μmol ml^{-1}), as internal standard, then centrifuged at 2000*g* for 15 min. An aliquot (500 μl) of each urine sample was transferred to an HPLC vial (22 × 12 mm) for automated analysis. Separation and detection of the non-conjugated metabolites (40 μl injection) was performed using the same chromatographic conditions described in method A. The metabolites 4CA, AACP, 4CAA, CNP and internal standard 4NP were eluted isocratically and metabolites 4CNB and 24DCA non-isocratically as follows: (0–30 min 100:0 v/v MP1:MP2; 31–45 min, 100:0 to 70:30 MP1:MP2; 46–59 min, 70:30 to 100:0 MP1: MP2; 60–68 min, 100:0 MP1:MP2) at 1 ml min^{-1}, 30°C with a detection wavelength of 240 nm.

Method C: Determination of NANPC

The conjugated metabolite NANPC and internal standard 4NP were analysed by HPLC coupled with UV. Briefly, NANPC and 4NP were eluted isocratically in mobile phase (80:20 v/v, 5 mM $NH_4H_2PO_4$ buffer (pH 6.0): MeOH) from urine 40 μl injection) at 1 ml min^{-1}, 30°C and detected at a wavelength of 340 nm.

Standard solutions for calibration of 4CNB and its metabolites in urine

Stock standard solutions of 10 mmol ml^{-1} 4CNB and its metabolitics were prepared separately in MeOH and stored under shielded light at −20°C for up to 4 weeks. On each day of analysis individual stock standards were combined to give 1 mmol ml^{-1} working standard solutions 1, 2 and 3, for calibration of metabolites in hydrolysed urine, non-hydrolsed urine, and NANPC respectively. Standard solution 1: 4CA, CNP, 4CNB and 24DCA; standard solution 2: AACP, 4CA, 4CAA, CNP, 4CNB and 24DCA; and standard solution 3: NANPC.

Serial dilutions were made from standard solutions and spiked into control urine (2 ml) with 20 μl of 4NP (100 μmol ml^{-1}) as internal standard, to give final working solutions of 0, 20, 50, 100 and 200 μmol ml^{-1}. These solutions (40 μl) were processed using the appropriate assay conditions for analysis of hydrolysed and non-hydrolysed metabolites present in urine.

Stability and recovery of 4CNB and its metabolites from acid-hydrolysed urine

Control urine was spiked with standard solution 1 to give metabolite concentrations of 50, 100 and 200 μmol ml^{-1} urine. Urine samples were treated under acid hydrolysis assay conditions described above. After base neutralization, 20 μl of 4NP (100 μmol ml^{-1}) was added to he urine and an aliquot (20 μl) removed and injected onto the HPLC-UV system described above. The ratio of metabolite peak area to 4NP internal standard was compared with the ratio obtained when the same standard solutions were spiked into urine and analysed under non-hydrolysed assay conditions method B. The recoveries of 4CA, CNP and 24DCA, at three different concentrations (50, 100 and 200 μmol l^{-1}), were greater than 90%. A lower recovery rate (approximately 80%) was obtained with 4CNB at each concentration analysed.

Precision of the assay

For NANPC, 4CA and CNP, the precision of the assay was calculated from six independent determinations, performed on three different 4CNB urine samples, from 4CNB-exposed workers. The average value of NANPC ± (relative SD) determined in each of these urines was 3.26 ± 0.065 (2%), 6.02 ± (1.6%) and 2.71 ± (5.8%) μmol g^{-1} creatinine. The average value of 4CA ± (relative SD) determined in each of these urines was 3.72 ± (11.7%), 2.21 ± (11.8)% and 1.26 ± (11.6%) μmol g^{-1} creatinine. The average value for CNP ± (relative SD) determined in each of these urines was 1.15 ± (14.8%), 0.71 ± (22.5%) and 1.45 ± (12.4%) μmol g^{-1} creatinine. The precision associated with detection of NANPC in non-hydrolysed urine, and 4CA or CNP in hydrolysed urine was calculated from multiple determinations of known concentrations of the standards spiked into control urine and taken through the appropriate assay procedure. From six replicates of 50, 100 and 200 μmol l^{-1} NANPC spiked into urine the average values ± the relative SD were 0.702 ± (7.7%), 1.526 ± (5.1%) and 3.28 ± (2.2%) μmol l^{-1}, respectively. From six replicates of 50, 100 and 200 μmol l^{-1} 4CA spiked into urine, the averaged values ± the relative SD were 0.574 ± (13.4%), 1.15 ± (10.1%) and 2.47 ± (0.8%) μmol l^{-1}, respectively. From six replicates of 50, 100 and 200 μmol l^{-1} CNP spiked into urine the average values ± the relative SD were 0.89 ± (11.3%), 1.76 ± (10.4%) and 3.85 ± (3.4%) μmol l^{-1}, respectively.

Calibration of urinary metabolites of 4CNB

The R^2 value calculated from the regression line of UV absorption plotted against concentration of each of the urinary metabolites of 4CNB was greater than 0.9.

Determination of creatinine levels in workers

Urinary creatinine levels were quantitated as published previously (Jones et al. 2005). The average concentration of creatinine in the exposed and control workers were 1.28 ± 0.86 and 1.05 ± 0.62 g l^{-1}, respectively, and ranged between 0.08 to 4.0 and between 0.33 and 2.20 g l^{-1}, respectively. Recent publications tend to report the data without creatinine correction (Barr et al. 2005). Only urine values with creatinine values between 0.3 and 3.0 g l^{-1} were considered. In the present study, five exposed workers had creatinine values < 0.3 g l^{-1} and one worker had creatinine

values $>3.0\ g\ l^{-1}$. The control workers had all creatinine values in between this range. Since we had no opportunity to obtain other urine samples from the workers, we included all the data. The data are reported in both formats in Tables II and III.

Air monitoring

Air monitoring of 2CNB, 3CNB and 4CNB was performed according to the NIOSH method 2005 (http://www.cdc.gov/niosh/nmam/pdfs/2005.pdf). Personal exposure monitoring (19 workers) was carried out using personal samplers from SKC Inc. (Eighty Four, PA, USA). The SKC pumps were pre-calibrated with two silica gel adsorbent tubes (SKC-product-Nr. 226-10) connected in parallel. The flow rate used was $200\ ml\ min^{-1}$. The samplers were attached to the lapels of the workers to monitor the breathing zone. Each set of tubes was exchanged after 2 h to prevent overloading and breakthrough of the adsorbed material. All silica tubes were desorbed (about 1 h) in 1 ml methanol (both front and back sections). Each sample (1 μl) was analysed by GC FID (HP 9897): temperature programme: 90°C increase at $10°C\ min^{-1}$ to 200°C. Column: Ultra 1 (crosslinked methyl siloxane, 50 m × 0.32 mm, 0.52-μm film thickness). Calibration lines were constructed using authentic standards; correlation coefficients of >0.99 and detection limits of $5\ \mu g\ ml^{-1}$ (10 ng injected) were achieved.

Results

Urine samples were collected from workers for the quantification of absorbed dose, resulting from recent exposure to 4CNB. Spot urine samples were voided from employees manufacturing chloronitrobenzene in China. The synthesis of 4CNB was performed by continual batch nitration of chlorobenzene with sulfuric acid and nitric acid. The major product being the *para*-substituted isomer 4CNB (70% yield). In total, 53 spot urine samples were collected from the 4CNB factory workers. Of these, 43 samples were from 38 exposed workers and ten were from non-matched controls. An additional eight urine samples were analysed from non-exposed volunteers, from the Department of Environmental and Occupational Medicine, Newcastle upon Tyne. Of the 43 exposed samples, 38 were post-shift samples, voided at the end of the 8-h work shift, and five were pre-shift samples, voided from a subset of workers, prior to commencement of their work shift.

For the determination of 4CNB and its metabolites in human urine we modified (see the Discussion section) the analytical protocol for analysis of rat urine developed by Yoshida (1993). Urine samples were analysed before and after acid hydrolysis to quantify both conjugated and non-conjugated forms of 4CNB and its metabolites. Baseline separation of urinary metabolites was achieved. The metabolites were detected by their UV absorbance at either 240 nm (AACP, 4CA, 4CAA, CNP, 4CNB and 24DCA) or 340 nm (NANPC). The retention times of these metabolites are presented in Table I. In each instance, 4NP was included as an internal standard to control for variation in the precision of the assay.

Three metabolites were detected in the post-shift urine samples from exposed workers. Each of the metabolites was excreted as a phase II conjugate. They were identified as the mercapturic acid, NANPC, conjugated 4CA and conjugated CNP

Table I. Chromatographic conditions used for the separation and detection of 4CNB and its metabolites from urine. The retention times of each compound are listed with the limits of detection (LOD), determined from standard aqueous solutions of each compound injected directly on column. The limits of quantitation (LOQ), calculated from standard solutions spiked into urine and taken through the assay procedure, are also listed. The LOD and LOQ were calculated at three times background noise (40 μl injected on column). The baseline resolution was achieved for each compound apart from CNP and 4CAA, which partially co-eluted under the chromatographic conditions described.

Method	Metabolite	Retention time (min)	LOD ($\mu mol\,l^{-1}$)	LOQ ($\mu mol\,l^{-1}$ urine)
A	4NP	11.4		
	AACP	15.5	0.70	1.0
	4CA	19.8	0.30	1.0
	CNP	26.3	0.15	0.6
	4CAA	27.9	0.20	3.0
	4CNB	31.9	1.00	5.0
	24DCA	50.3	0.50	1.0
B	4NP	10.9		
	NANPC	6.1	0.10	0.5
C	4NP	11.4		
	4CA	19.7	0.30	1.0
	CNP	26.3	0.15	0.6
	4CNB	31.9	1.00	7.0
	24DCA	50.2	0.50	1.0

(Figures 2 and 3). Other metabolites listed in Table I could not be found within the LOQ of the assay.

NANPC was the only metabolite detected in non-hydrolysed urine. The metabolite was characterized by its UV absorption at 340 nm and its retention time (6.1 min) with respect to authentic standard (Figure 2). The metabolite NANPC was detected in all post-shift urine samples from the exposed workers ($n=38$). The concentration range of NANPC detected in these urines was 0.3–34.3 $\mu mol\,l^{-1}$ (Table II). The per cent difference between duplicate analyses ranged between 2.1 and 6.1%. The remaining two metabolites (4CA and CNP) found in urine from exposed workers were present as conjugates, and were only detected as cleavage products following acid hydrolysis of the urine. The cleavage products were characterized by their UV absorption at 240 nm, and retention time, with respect to authentic standards. The cleaved metabolite 4CA (19.7 min) eluted prior to CNP (26.3 min) and was detected in 29 of the 38 post-shift urine samples from exposed workers. A typical UV chromatogram of 4CA and CNP detected in hydrolysed post-shift urine is presented in Figure 3. The range of 4CA detected in these urines was from not detected to 10.5 $\mu mol\,l^{-1}$. The percent difference between duplicate analyses ranged between 4.5 and 10.1%. The cleavage product of the phase II conjugated metabolite, CNP, was detected in 36 of the 38 post-shift urine samples from exposed 4CNB workers. The range of CNP detected in these urines was from not detected to 11.8 $\mu mol\,l^{-1}$. All values determined for NANPC, 4CA and CNP were an average calculated from two independent determinations. The percent difference between determinations ranged between 5.2 and 11.3% NANPC, 4CA and CNP were not detected in the post-shift urine taken from Chinese non-exposed control workers, or in the Newcastle volunteers, within the LOQ of the assay. NANPC, 4CA and CNP were below the LOQ in a subset of pre-shift urine samples ($n=5$) voided from the same workers.

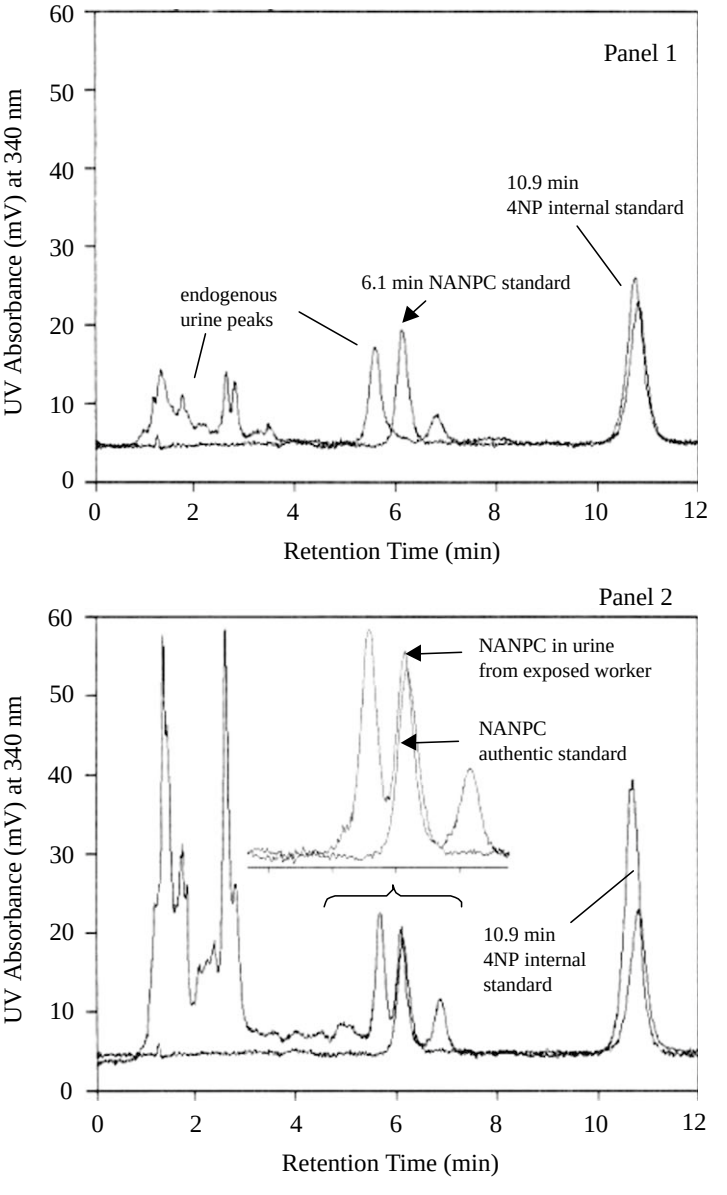

Figure 2. RP-HPLC coupled with UV detection (340 nm) for the determination of NANPC in non-hydrolysed urine. Constituents of urine were separated isocratically using the HPLC method C. Non-hydrolysed urine (40 µl), containing 4NP internal standard, from control (Panel 1) and exposed (Panel 2) 4CNB workers were injected onto the RP-HPLC-UV system. A solution containing the authentic standard NANPC (6.1 min) and internal standard 4NP (10.9 min) was run under identical conditions and has been included for comparative purposes.

Hydrolysis of 4CA-N-glucuronide and 4COA

The assay conditions for complete hydrolysis of the conjugate forms of 4CA were investigated. The increase of 4CA with time can be seen in Figure 4. The peak area of 4CA released form 4COA, with respect to 4NP internal standard, was plotted against

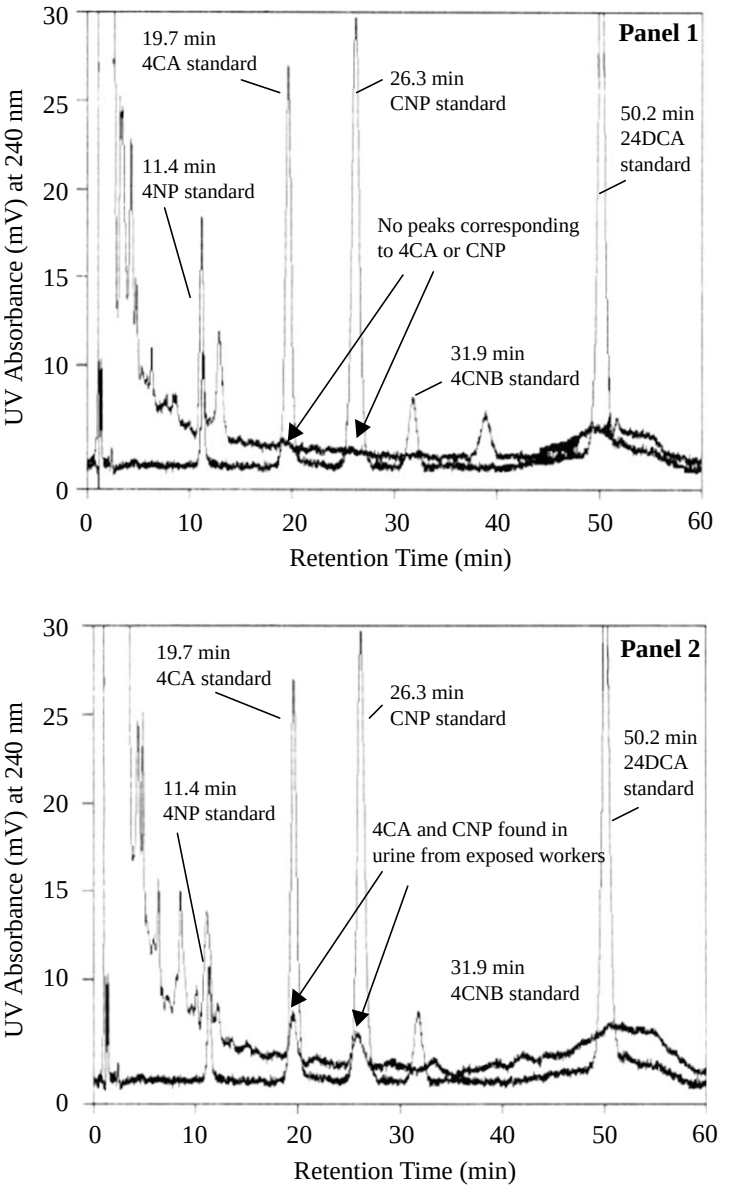

Figure 3. RP-HPLC coupled with UV detection (240 nm) for the determination of urinary metabolites 4CA, CNP, 4CNB and 24DCA in hydrolysed urine. The metabolitics and 4NP internal standard (100 μmol l^{-1}) were separated by gradient elution using the HPLC method B. Panel 1 and 2 show metabolites detected in hydrolysed urine (40 μl injection) from control (Panel 1) and exposed (Panel 2) 4CNB workers, respectively. A standard solution of authentic standards (50 μmol l^{-1}) has been included for the identification of each metabolite. The metabolities 4CA and CNP were detected only in urine from exposed workers.

time (data not shown). The level of 4CA released from 4COA did not increase after 120 min, under the described assay conditions, suggesting complete hydrolysis had occurred. With respect to 4CA-N-gluc, the glucuronide was very unstable in acid and had fully cleaved at time point zero (on addition of concentrated HCl) with no

Table II. Urine metabolites of 4CNB found in exposed workers.

Concentration unit	4CA, mean ± SD (25^{th}, 50^{th}, 75^{th})	CNP, mean ± SD (25^{th}, 50^{th}, 75^{th})	NANPC, mean ± SD (25^{th}, 50^{th}, 75^{th})
μmol g^{-1} creatinine	2.83 ± 4.38	4.66 ± 6.74	6.95 ± 4.39
	(nd[a], 1.38, 3.27)[b]	(1.83.2.96. 6.28)[b]	(3.97, 6.26, 8.58)[b]
μmol l^{-1} urine	2.38 ± 2.36	3.91 ± 2.97	8.61 ± 8.34
	(nd[a], 2.00, 3.44)[b]	(1.59, 3.30, 5.07)[b]	(2.57, 6.19, 10.12)[b]

nd[a], Not detected, below the LOQ; [b]Percentiles.

further increases in the amount of 4CA released for time points 5, 15, 30, 60, 210 or 240 min.

A subset of 4CNB post-shift urine samples from exposed ($n = 8$) and control ($n = 4$) workers were examined for the presence of 4COA and 4CA-N-gluc under hydrolysed and non-hydrolysed assay conditions. Preliminary experiments showed that a peak, which has tentatively been assigned as 4COA, was present in non-hydrolysed urine from the exposed workers and disappeared following acid hydrolysis, when analysed by HPLC coupled with UV detection at 240 nm (Figure 5). This peak was present in non-hydrolysed urine from exposed 4CNB workers but was absent in urine samples from control workers. The peak eluted very early, under the chromatographic

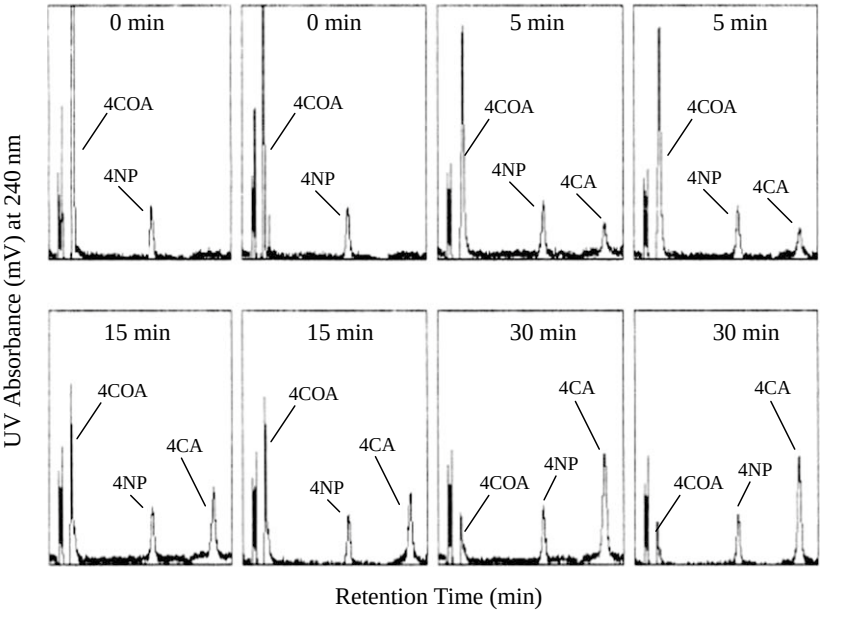

Figure 4. Increase of 4CA released from 4COA, relative to 4NP, over the time course of 0–30 min. RP-HPLC coupled with UV detection for the analysis of 4CA released from acid hydrolysis of 4COA. Aqueous solutions (40 μmol l^{-1}) of 4COA (2 ml), spiked with 4NP internal standard (1 mmol/2 ml) were hydrolysed in concentrated HCl (0.1 vols) at 100°C for the designated time points 0, 5, 15, 30, 60, 120 and 240 min. The hydrolysates were neutralized with base then analysed by HPLC coupled to a UV detector and detected at 240 nm, using method A. The peak area of 4CA relative to 4NP was plotted against time. The data points were averaged from four independent determinations ± SD (data not shown).

conditions described (2.7 min). There were a number of background peaks that potentially interfered with detection of 4COA at this elution window. A peak was found in control urine at 2.5 min, which partially co-eluted with the 4COA authentic standard (Figure 5, panel 1).

Air levels of CNB

The mean 8-h TWA exposure levels for 2CNB, and 4CNB were 0.49 ± 0.32 and 1.17 ± 0.92 mg m^{-3}, respectively. The mean levels of 2CNB were below and the mean

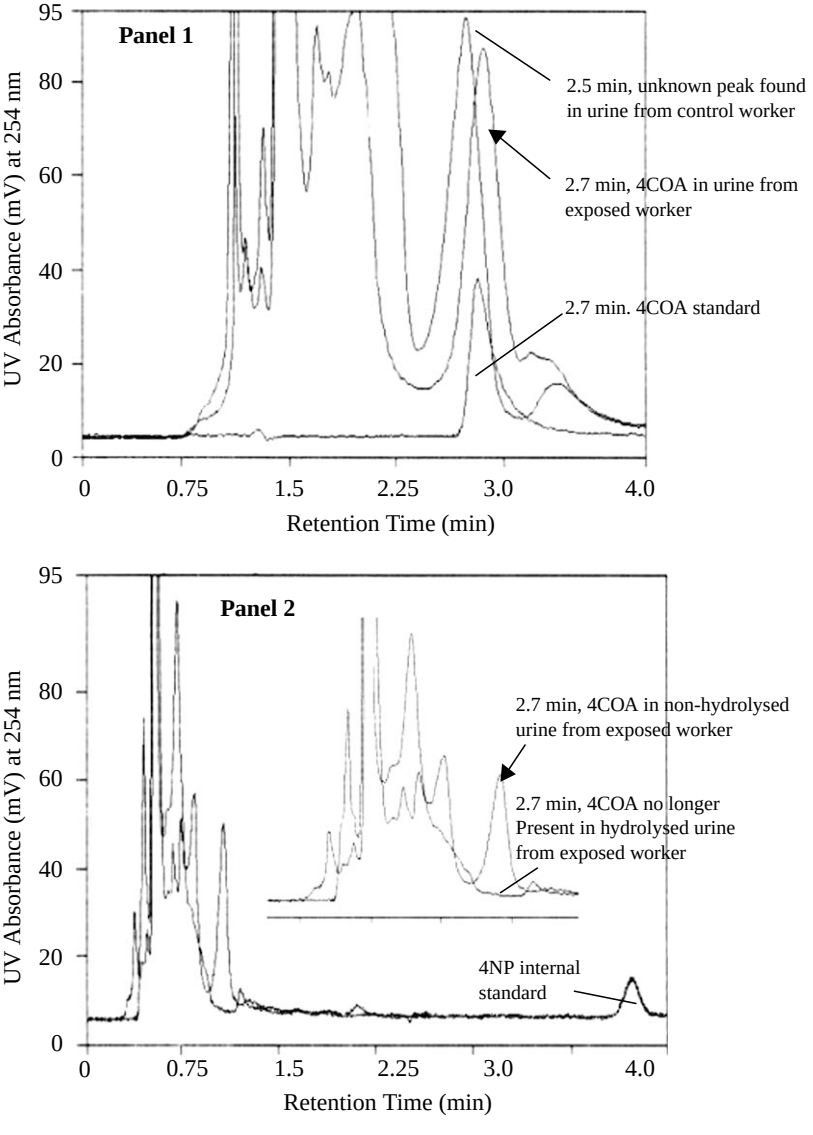

Figure 5. RP-HPLC coupled with UV detection (240 nm) for determination of 4COA in non-hydrolysed urine. Panel 1 shows non-hydrolysed urine (20 µl) from control and exposed 4CNB workers, and 4COA authentic standard. Panel 2 shows non-hydrolysed and hydrolysed urine from a worker exposed to 4CNB.

levels of 4CNB were above the OSHA PEL and the UK exposure limits set at 1 mg m^{-3}. Two different laboratories (Newcastle and Beijing) determined the air level of 4CNB. The log-transformed values for 4CNB correlate with $r = 0.87$ ($p < 0.01$).

Correlation between urinary metabolites and other exposure markers

Initially we investigated correlations between the different urinary metabolites, 4CA, CNP and NANPC, detected in each post-shift urine to ascertain whether a positive association existed between each of the separate biomarkers, such that one metabolite could be used as a means of predicting the levels of the other metabolites. The correlations between the urinary metabolites 4CA, CNP and NANPC were performed with raw data and with the data corrected with creatinine (Table III). There was a positive association between each of the metabolites correlated such that an increase in one metabolite was accompanied by an increase in each of the other metabolites. In general the correlations were better with the raw data. In addition, differences between the two methods of correlations used (Spearman rank order and Pearson) were smaller in the case of the raw data. This might be explained by the fact that for the creatinine-corrected data two metabolites (CNP, 4CA) were not normally distributed. In contrast with the raw data only NANPC was not normally distributed. There was a moderate correlation between NANPC and both 4CA and CNP. The correlation between 4CA and CNP was minor. The correlation between the total metabolites and both 4CA and CNP were good. The best correlation was found between the total metabolites and NANPC. There is a moderate inverse correlation between age and the creatinine levels (Table III). The raw metabolite levels of CNP and NANPC decreased with age. This correlation disappeared for CNP when the creatinine-corrected values were used. There was a good correlation between the total metabolities and the creatinine levels. The creatinine levels increase with the level of internal exposure. Except for the creatinine adjusted 4CA levels, there was no correlation between the air levels and the urinary metabolites. Except for CNP, all urine metabolite levels correlate with the haemoglobin adduct levels (Jones et al. 2006) of 4CNB (Hb-4CA).

Comparison of urinary metabolites and health effects

A full medical examination was performed on each worker. Health effects such as fatigue, dizziness, headache, eye irritation, splenomegaly, cardivascular effects (ECG1, ECG2, ECG3), insomnia, and dyspnea, have been linked to exposure to CNB (Hazardous Substances Data Bank, http://toxnet.nlm.nih.gov). Evidence of these health effects, and the clinical parameters red and white blood cells (RBC, WBC), protein, bilirubin, and glucose in urine were compared with the urinary metabolites expressed as raw concentrations or as creatinine corrected values. In total 45 comparisons were made with the three metabolites. In 19 cases an opposite trend of the metabolite level was seen with respect to the health effects, depending on the concentration unit, which was taken. Therefore the prediction of the health effect using the urinary concentrations was ambiguous. The median metabolite concentration expressed as $\mu mol\,l^{-1}$ were higher in 23 of 45 negative effects. The median creatinine adjusted metabolite levels were higher in 34 of 45 negative effects. Therefore it appears that creatinine adjusted metabolite levels are a better marker for negative effects. The median levels of 4CA in urine expressed as ($\mu mol\,g^{-1}$

Table III. Spearman rank and Pearson correlations of urine metabolites with air level, age, creatine and Hb-adducts in exposed workers. According the one-sample Kolmogorov–Smirnov test all data were normally distributed except for NANPC (μmol l^{-1}), CNP (μmol g^{-1} creatinine) and 4CA (μmol g^{-1} creatinine).

	4CA	CNP	NANPC	Creatinine	Age	Air-4CNB	Hb-4CA[e,f]
U-tot[a]							
r (ranks)	0.73[b] (0.72[b])	0.75[b] (0.64[b])	0.96[b] (0.82[b])	0.70[b]	−0.40[e] (−0.21[d])	0.03[d] (0.09[d])	0.57[b]
r (Pearson)	0.80[b] (0.77[b])	0.72[b] (0.92[b])	0.97[b] (0.78[b])	0.77[b]	−0.45[b] (−0.33[c])	−0.30[d] (−0.03[d])	0.57[b]
4CA[a]							
r (ranks)		0.33[c] (0.22[d])	0.63[b] (0.50[b])	0.32[c]	−0.16[d] (−0.03[d])	0.25[d] (0.29[d])	0.51[b]
r (Pearson)		0.41[c] (0.59[b])	0.73[b] (0.39[c])	0.48[b]	−0.25[d] (−0.20[d])	0.00[d] (0.49[c])	0.52[b]
CNP[a]							
r (ranks)			0.68[b] (0.38[c])	0.56[b]	−0.38[c] (−0.17[d])	0.14[d] (0.14[d])	0.27[d]
r (Pearson)			0.56[b] (0.61[b])	0.65[b]	−0.41[c] (−0.29[d])	−0.25[d] (−0.08[d])	0.27[d]
NANPC[a]							
r (ranks)				0.78[b]	−0.37[c] (−0.28[d])	−0.08[d] (−0.30[d])	0.55[b]
r (Pearson)				0.74[b]	−0.43[b] (−0.35[c])	−0.27[d] (−0.35[d])	0.58[b]
Creatinine							
r (ranks)					−0.35[c]	−0.15[d]	0.30[d]
r (Pearson)					−0.37[c]	−0.31[d]	0.35[e]

[a]Values in parentheses correspond to the correlations with the values (μmol g^{-1} creatinine). The other urine values are expressed as μmol l^{-1}.
[b]$p < 0.01$; [c]$p < 0.05$; [d]$p > 0.05$; [e]Hb-4CA: Hb-adduct of 4CNB (Jones et al. 2005); [f]no correlation with the creatine-adjusted urine metabolite values, except for NANPC.

creatinine) was higher for all negative effects except for headache. For CNP all negative effects were found at higher concentrations except for fatigue, headache, dyspnea, glucose in urine and RBC in urine. For NANPC the negative effects were found at higher concentrations except for headache, insomnia, dyspnea, rbc in urine and glucose in urine. Therefore negative effects of 4CNB were reflected the best by the 4CA levels in urine.

Discussion

Validation and development of the analytical procedure

Analysis of the potential metabolites in the urine from workers exposed to 4CNB was performed using the analytical procedure described by Yoshida (1993). The assay had been used for the quantification of 4CNB and its metabolites in rat urine, but had not been applied to the analysis of urine from humans. The chromatographic conditions described by Yoshida (1993) for the separation and detection of each metabolite in hydrolysed and non-hydrolysed urine were time consuming. In total, five chromatographic runs were required per sample, which in turn meant a run time of 8 h per sample. Additionally, the method lacked an internal standard to control for sample dilution errors associated with the different stages of the assay, such as neutralization of the urine acid hydrolysate. The procedure was optimized and under the newly devised conditions it was possible to achieve baseline resolution of each metabolite, as well as separation from any interfering peaks in hydrolysed and non-hydrolysed human urine, using three chromatographic runs. This dramatically reduced the analysis run time per sample (2 h 20 min). Under these new chromatographic conditions the LOQ of the assay (Table I) were still comparable with those previously described (Yoshida 1993). As the workers were also exposed to 2CNB, it is possible that its metabolites were also present in peaks observed. Since 2CNB levels were 2.2 times lower than 4CNB, however, its metabolites would be below the LOQ in most cases. Furthermore, if the metabolism previously noted in rabbits is an indication, a larger variety of metabolites are expected for 2CNB than for 4CNB (Bray et al. 1956).

Metabolism studies for 4CNB in rat, and acutely exposed humans have shown that ring-hydroxylated metabolites of 4CNB were excreted in urine as glucuronide, and possibly sulfate conjugates (Yoshida et al. 1991, 1992, 1993). Therefore optimization of hydrolysis conditions for cleavage of these conjugates was a major consideration in the assay procedure. A subset of urine samples from exposed workers were analysed following acid hydrolysis according to Yoshida's method (Yoshida 1993). None of the expected ring hydroxylated metabolities was observed (data not presented). Therefore acid hydrolysis conditions to ensure complete cleavage of potential glucuronide and sulfate conjugates were investigated to determine whether the absence of metabolites was due to suboptimal hydrolysis conditions, or that metabolites were below the LOQ of the assay. The glucuronide and sulfate conjugates of 4NP were used as surrogates for potential conjugates excreted in the urine resulting from metabolism of 4CNB. The time-course plot confirmed that overnight hydrolysis at 100°C was required to cleave fully the 4NP-gluc in contrast to 5 min for 4NP-sulfate.

Metabolism of 4CNB in chronically exposed workers

The current study was designed to reflect the recent internal exposure dose of 4CNB by measuring 4CNB and its metabolites in urine, and to determine whether the metabolic fate of 4CNB in these workers differed to observations made in cases of acute human poisoning (Yoshida et al. 1992, 1993). The results presented in Table II confirmed that 4CNB had been absorbed, metabolized and execrated in the urine of workers exposed to 4CNB. It was possible that exposure had occurred by inhalation and dermal routes. Metabolites of 4CNB were found only in post-shift urines from exposed workers. No metabolites were detected in urine from factory control workers. Therefore determination of urinary metabolites of 4CNB provided an effective marker of recent occupational exposure to 4CNB. There were no metabolites detected in the pre-shift urine samples taken from a subgroup of exposed workers, which suggested that the absorbed dose per work-shift had been excreted to levels below the LOQ of the assay within 24 h. In contrast, for acutely poisoned workers (Yoshida et al. 1993), disappearance of 4CNB from the body took considerably longer and decreasing levels of 4CNB were found in each subject's urine ($n=6$) over the course of their hospitalization (30 days). The levels of metabolites in the acutely poisoned subjects were approximately 1000-fold higher than the levels reported in this study for chronically exposed workers. The half-lives of each of the metabolites detected in acutely exposed humans and the rat were reported to be several hours. The rates of disappearance of identical metabolites in the rat were shown to decrease with increasing dose, suggesting saturation of clearance mechanisms. Therefore the amount of 4CNB absorbed may explain the difference in excretion between the chronically exposed and acutely poisoned workers.

All of the absorbed 4CNB had been metabolized in the chronically exposed workers prior to excretion, as no unchanged 4CNB was detected in any of the urine samples. This agreed with the data reported for acutely poisoned workers (Yoshida et al. 1993). For rats dosed intraperitoneally, only trace amounts of unchanged 4CNB were detected in the urine. These results indicated that metabolism was the major route of elimination *in vivo*.

Three conjugated metabolites were identified in the urine of chronically exposed workers. Two of the metabolites were detected as their respective cleavage products (CNP and 4CA) following acid hydrolysis of the conjugates, and one (NANPC) was detected as a conjugate in non-hydrolysed urine. No free metabolites were excreted in urine from exposed workers. In chronically exposed workers 4CNB was excreted in urine through three distinct metabolic pathways: reduction of the nitro group of the parent compound, ring hydroxylation and conjugation with sulfate/glucuronide, or glutahione conjugation through substitution of the chloro moiety, resulting in formation of the mercapturic acid.

The mercapturic acid, NANPC was the predominant metabolite identified in urine. NANPC excreted in post-shift urine of chronically exposed workers ranged between 39 and 59%. In two workers NANPC was the only metabolite detected, whereas in one worker NANPC was responsible for only 9% of the excreted dose of 4CNB. In acutely poisoned workers Yoshida et al. (1993) the fraction of the absorbed dose excreted as the mercapturic acid ranged between 44 and 53% ($n=6$). In rats dosed intraperitoneally with 4CNB, approximately two-thirds of the dose was excreted in the urine. NANPC was the major metabolite, and accounted for 46% of the total metabolites detected (Yoshida et al. 1991, Yoshida 1994). The rat study indicated that

metabolism of 4CNB to NANPC was linear over the dose range studied, which inferred that excretion of NANPC was proportional to the absorbed dose of 4CNB. Consequently, urinary NANPC was considered as a suitable biomarker in urine for measuring recent exposure to 4CNB in rats and humans.

The two remaining conjugates, detected as cleavage products in hydrolysed urine, were CNP and 4CA. It is likely that the conjugate forms of CNP and 4CA eluted at the solvent front with other polar compounds. In non-hydrolysed urine CNP and 4CA were identified as important metabolites in the biotransformation of 4CNB in acutely exposed humans and rats. The conjugate of CNP was likely to be either the glucuronide or sulfate since cleavage of the CNP-conjugate required intense hydrolysis conditions (0.1 vol. concentrated HCl, 100°C, 24 h). This supported the view that the conjugate was a glucuronide since cleavage of a surrogate glucuronide (4NP-gluc) required similar hydrolysis to those of the CNP-conjugate. In contrast, the 4NP-sulfate was relatively labile under acid hydrolysis conditions. The percentage of conjugated CNP excreted in the urine, relative to the total level of metabolites detected, was approximately 30%. Between workers this level ranged from 15 to 41%. In six workers the CNP conjugate was the principal metabolite excreted but was not detected in two workers and accounted for less than 12% of the total metabolites detected in a further six workers. In acutely exposed workers, conjugated CNP accounted for approximately 12% of the excreted metabolites and ranged between 5 and 17%. Interestingly, in rat, excretion of CNP-conjugates accounted for 16.7% of the excreted metabolitics (Yoshida 1994). In rat, excretion of the CNP conjugate was considered to be non-linear over the dose range studied. At the highest dose the CNP-conjugate accounted for only 8% of total metabolites excreted.

The results presented in Table II show that the reduction pathway in chronically exposed workers was not as prevalent. Conjugated 4CA was detected in 29 of the 38 post-shift urine samples from the exposed workers.

The conjugated metabolite of 24DCA had only been recently described following acute exposure to 4CNB in man (Yoshida et al. 1992) and in rat (Yoshida et al. 1991, 1993). It was suggested that chlorination of 4CA was catalysed by a myeloperoxidase present in activated leucocytes as a direct consequences of the toxicity of the absorbed 4CNB. Therefore, in these instances the metabolite may have been a direct result of toxicity caused by acute exposure. The metabolite levels in the acutely poisoned workers were 1000-fold higher than the levels reported herein for the chronically exposed workers. Therefore it could be inferred from the above that the absorbed dose in chronically exposed workers was insufficient to cause systemic toxicity and hence activation of the chlorination biotransformation pathway.

In the present study the acetylated metabolite, 4CAA and AACP were not observed in non-hydrolysed urine from the workers chronically exposed to 4CNB. Experiments addressing the stability of 4CNB and its metabolites in urine (data not shown) confirmed that 4CAA was stable in urine at −20°C. These results indicated that acetylation had not occurred or was a very minor pathway for the conjugation of 4CA in these workers. Indeed, Yoshida et al. (1992) reported that excretion of acetylated metabolites may be a relatively minor pathway for the elimination of chloroanilines in humans, and may be absent in some individuals.

The possibility that the acetylated metabolite, 4CAA, was hydroxylated to the glycolanilide and further oxidized to the oxanilic acid prior to excretion was considered. Previously, this pathway was shown to be significant in the metabolism

of 4CA in rat, mouse and monkey (Ehlhardt & Howbert 1991). An authentic standard of this metabolite was synthesized and initial results suggested that the 4-chloro-oxanilic acid (4COA) might have been formed in the chronically exposed workers (Figure 5). A peak was seen in non-hydrolysed urine from exposed workers that eluted near the solvent front. The peak disappeared following acid hydrolysis, under conditions described previously for the release of 4CA. This peak had an identical retention time to the synthesized 4COA standard. Additionally, the profile for release of 4CA from the 4COA standard, under acid hydrolysis conditions, was similar to the conditions required for cleavage of the unknown conjugate in the urine. There did appear to be an interfering peak that partially co-eluted with 4COA. This peak was seen in some of the control and some of the exposed workers. Therefore the phase II conjugate of 4CA has been tentatively assigned as the chloro-oxanilic acid.

The alternative to the chloro-oxanilic acid conjugate was the N-glucuronide of 4CA, 4CA-N-gluc. There was evidence in the literature to suggest that N-glucuornidation might be a major conjugating reaction, competing with acetylation and sulfation, in the excretion of 4CA (McCarthy et al. 1985). Therefore the amine 4CA-N-gluc was synthesized to see if it could be identified in the urine of workers chronically exposed to 4CNB. The standard eluted at a retention time too close to the solvent, under the described chromatographic conditions, was identified by UV absorption. However, the N-glucuronide was discounted as a possible phase II conjugate of 4CA based on the results from hydrolysis experiments with 4CA-N-gluc (data not presented). These experiments indicated that 4CA-N-gluc was extremely labile under acidic conditions and cleaved almost instantaneously on addition of concentrated HCl, as shown previously for N-glucuronide of 4-aminobiphenyl (Babu et al. 1996), and benzidine (Zenser et al. 1999). This did not fit in with the stability profile of the 4CA-conjugate determined in the urine of exposed workers.

Conclusion

Correlations between the different metabolities identified a positive association between NANPC and both the conjugate forms of 4CA and CNP in urine, which were statistically significant ($p < 0.001$). NANPC reflected the levels of 4CA and CNP and was the most prevalent metabolite detected in all the exposed workers (but it was absent in controls). Therefore, it was the most appropriate biomarker in the urine for a recent absorbed dose. The absence of a correlation between the air exposure levels and the absorbed dose of 4CNB, which more accurately reflected the toxicologically active dose, was of concern since occupational hygiene standards in the UK for 4CNB have been based primarily on 8-h TWA exposure limits. This raises the question of whether it would be more appropriate to set a biological exposure index.

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