

Biological monitoring for occupational exposures to *ortho*-toluidine and aniline

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In 1988, epidemiologists at the National Institute for Occupational Safety and Health (NIOSH) conducted an investigation at a plant producing an antioxidant and an accelerator used in tire manufacturing. An excess of bladder cancers among plant workers was found and attributed to *ortho*-toluidine (*o*-toluidine) and aniline exposures, two of the principal chemicals used in the production processes (1, 2). NIOSH conducted a second study with biological monitoring at this plant in 1990 to investigate worker exposures to *o*-toluidine and aniline. The biological monitoring methods developed for the 1990 study and the results of the analyses completed to date on the collected samples are presented in this report.

A two-pronged effort was undertaken for determining exposures to these aromatic amines, namely, the traditional biological monitoring approach of urinalysis and the analysis of blood for protein adducts serving as biomarkers of internal dose. Urinary monitoring of parent compounds or metabolites provides an estimate of the recent uptake of chemicals and is especially important for exposure assessment when the potential for skin absorption exists, as is the case for *o*-toluidine and aniline in this study. Conversely, protein adduct measurements provide an index of cumulative dose and often serve as dosimeters for carcinogen-deoxyribonucleic acid adducts residing in inaccessible target tissue. The blood protein adducts are generally present for the lifetime of the protein to which the carcinogen (or metabolite) is covalently adducted. Since the life-span of a human red blood cell is 120 d, the level of adducted hemoglobin reflects the average exposure over four months. Serum albumin

has a half-life of 20-25 d in humans. On the assumption that adduct half-life would parallel albumin half-life, after a single exposure adducted albumin could still be present up to 125 d, on the basis of first-order kinetics and five half-lives for complete clearance.

Methods

Urinary biological monitoring. Paramount considerations in biological monitoring are the medium to be analyzed, the timing of the collection of the samples, and the compounds for which to analyze. In humans, aniline is rapidly metabolized and excreted in the urine (3). Rat data suggest that *o*-toluidine likewise is rapidly metabolized and excreted (4). Thus biological monitoring using pre- and postshift urine samples to detect exposure during that workshift seems appropriate. While ring hydroxylation, followed by sulfate and glucuronide conjugation, is the major metabolic pathway for both aniline in humans (3) and *o*-toluidine in rats (4), small levels of the free amines are also present in urine. This occurrence suggests that analysis for these compounds is feasible.

Because we expected to collect and analyze a large number of samples, we hoped to identify a single method for sample preparation and analysis which would allow simultaneous determination of both analytes. El-Bayoumy et al (5) quantified *o*-toluidine and aniline at the parts-per-billion level in urine from smokers and referents with no known occupational exposures. However, their methodology for sample work-up and analysis was too labor intensive for use with large numbers of samples. Consequently, we developed an alternative analytical procedure. Our method quantifies not only any unmetabolized aniline and *o*-toluidine present in urine, but also the metabolites acetanilide and *N*-acetyl-*o*-toluidine, which are converted by base hydrolysis back to the parent amines. In this procedure, sodium hydroxide was added to a 4-ml aliquot of urine until it was 4.7 M. The resulting solution was then heated at 80°C for 2 h. The neutral and basic compounds were extracted from this hydrolysate with butyl chloride, the basic compounds subsequently being back-extracted from the butyl chloride solution with 0.1 N hydrochloric acid. An aliquot of the acidic extract was subjected to ion-interaction

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reversed-phase high-performance liquid chromatography (HPLC) on a 30-cm $\times$ 6-mm column of Nova-pak C18 with coulometric electrochemical detection. The mobile phase was 60 mg $\cdot$ l⁻¹ sodium dodecyl sulfate and 53 mM PO₄³⁻ (pH 3.3) in 40% methanol:water (v:v).

Biomarkers of internal dose. The timing of the sample collection for the quantitation of protein adducts is less critical than for urinary biological monitoring. Lewalter & Korallus (6) have shown the presence of aniline-hemoglobin adducts in humans exposed to aniline in an industrial accident. Adduct levels peaked at 24 h and were still detectable 7 d later. Stillwell et al (7) found an increase in the formation of *o*-toluidine-hemoglobin adducts for smokers in comparison with nonsmokers (100 versus 34 pg *o*-toluidine $\cdot$ g⁻¹ hemoglobin) after base hydrolysis of the adducted hemoglobin. However, the technology used for quantifying the parent amine (*o*-toluidine) by these investigators, gas chromatography-mass spectrometry (GC-MS) utilizing negative ion chemical ionization detection, is not available in many laboratories. Consequently, we developed two alternative analytical procedures, one utilizing HPLC and a second employing gas chromatography (GC), to analyze base-hydrolyzable protein adducts. In vivo and in vitro studies were conducted to facilitate the development of these analytical methods and to characterize the formation of *o*-toluidine-protein adduct. Only *o*-toluidine was investigated in the animal model, since it has been shown to be carcinogenic in rats. However, both procedures were capable of concurrent analysis for both aniline and *o*-toluidine in the same sample.

Rat or human hemoglobin or albumin was incubated with 130 nmol of [¹⁴C]*o*-toluidine at 37°C in a system consisting of rat liver microsomes and cofactors for 10, 20, 30, 45, or 60 min. Hemoglobin or albumin was isolated and the ¹⁴C levels were determined by liquid scintillation counting. Bound *o*-toluidine was quantified on the assumption that all bound radioactivity represented parent *o*-toluidine. For induction studies, male Sprague-Dawley rats were pretreated by intraperitoneal injection with 100 mg phenobarbital $\cdot$ kg⁻¹ body weight daily for 3 d, 80 mg beta-naphthoflavone $\cdot$ kg⁻¹ body weight at 48 h, or no treatment prior to sacrifice and the preparation of microsomes.

The dose-effect for *o*-toluidine-hemoglobin adduct formation was tested in rats through the administration of a single intraperitoneal injection of [¹⁴C]*o*-toluidine at 10, 20, 40, 50, or 100 mg $\cdot$ kg⁻¹ body weight with sacrifice at 4 h. Blood was collected and hemoglobin isolated (8). Protein-bound ¹⁴C was determined by liquid scintillation counting. For the analysis of adducted *o*-toluidine, hemoglobin was hydrolyzed with 1 N sodium hydroxide for 1 h and extracted with ethyl acetate. HPLC analysis was performed with the use of automated precolumn *o*-phthalaldehyde

derivatization prior to methanol:water separation (8). The separated components were detected by fluorescence (ex = 240 nm, em = 340 nm) and quantified with 4-ethylaniline as an internal standard. For the GC analysis, the ethyl acetate extracts were back-extracted into 1 N hydrochloric acid and concentrated. The samples were redissolved in benzene and derivatized with an excess of pentafluoropropionylimidazole. The derivatization procedure was carried out overnight at 60°C and terminated by the addition of methanol. The samples were analyzed by temperature-programmed GC on a 30-m $\times$ 0.32-mm inner diameter column coated with a 0.5- μ m thick film of Supelcowax[®] 10. Separated components were detected by electron capture (EC) and quantified with 4-ethylaniline as an internal standard.

Field samples for analysis. Pre- or postshift urine samples or both were obtained from 53 exposed and 36 unexposed workers. A 50-ml aliquot of each was stabilized with 5 g of citric acid, shipped to the lab on dry ice, and stored at -68°C until the analysis. The 209 samples submitted from the field also included 20 quality-control samples and duplicate 50-ml aliquots of 17 samples from the workers.

A total of 84 whole-blood samples was received for adduct determinations. The red blood cells were immediately washed and stored at 4°C prior to the isolation of hemoglobin. The hemoglobin was isolated by ethanol precipitation and stored at -80°C until the analysis.

Results

Urinary biological monitoring. Data for the recovery and precision of the method, as determined by the analysis of spiked urine samples prepared in the laboratory, are given in table 1. These spiked samples were interspersed and analyzed with the field samples. A plot of recovery as a function of storage time suggested

Table 1. Method recovery and precision using spiked urine samples.

Compound	Nominal concentration ^a (μ g $\cdot$ l ⁻¹)	N ^b	Recovery (%)		
			Average	Relative standard deviation	Range
Aniline	6.8	10	109	26	74–176
Aniline	18	10	97	14	72–111
Aniline	77	10	93	12	82–106
Acetanilide	19 ^c	13	96	16	68–116
<i>o</i> -Toluidine	4.2	10	101	32	64–183
<i>o</i> -Toluidine	20	10	93	17	75–125
<i>o</i> -Toluidine	102	10	86	14	65–98
<i>N</i> -Acetyl- <i>o</i> -toluidine	16 ^c	13	83	17	57–106

^a The nominal value is the concentration of added compound plus the background concentration.

^b Number of data points.

^c Expressed in terms of the free amine.

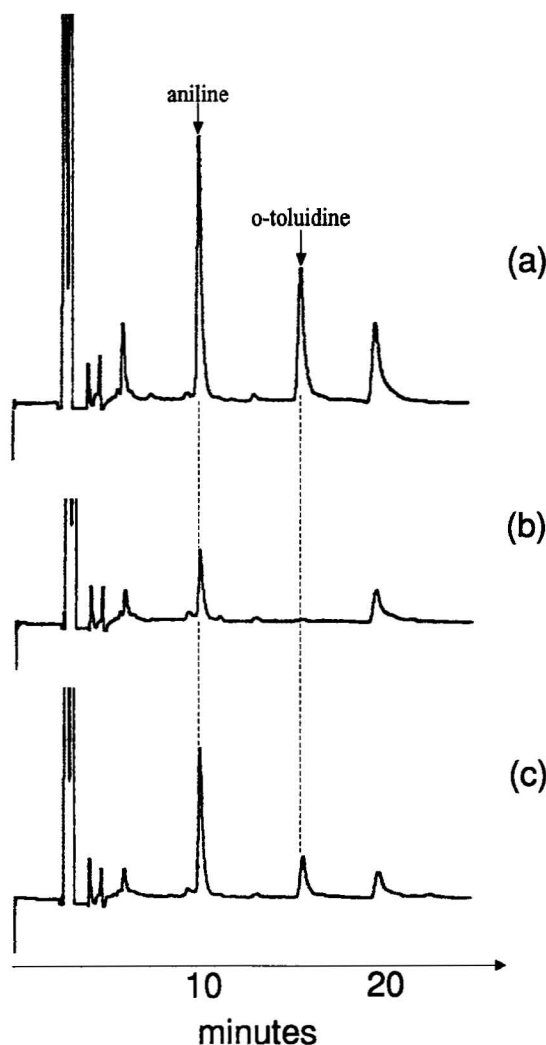


Figure 1. Typical chromatograms: (a) field urine sample — $14.3 \mu\text{g aniline} \cdot \text{l}^{-1}$ and $11.8 \mu\text{g o-toluidine} \cdot \text{l}^{-1}$ found; (b) urine sample from an unexposed individual — $4.0 \mu\text{g aniline} \cdot \text{l}^{-1}$ found; (c) same as figure 1b with — $4.0 \mu\text{g aniline} \cdot \text{l}^{-1}$ and $4.0 \mu\text{g o-toluidine} \cdot \text{l}^{-1}$ added.

that no *o*-toluidine or aniline was lost from the samples during storage at -68°C over a seven-month period. Using data from the analysis of 18 urine samples fortified to 1.4 – $15 \mu\text{g} \cdot \text{l}^{-1}$ plus five unfortified urine samples, we estimated the limit of detection to be $0.6 \mu\text{g} \cdot \text{l}^{-1}$ in urine. Similarly, the limit of detection for aniline was estimated to be $1.4 \mu\text{g} \cdot \text{l}^{-1}$ in urine.

Typical chromatograms are shown in figure 1. The results from the analyses of the field samples are summarized in table 2. In addition to the 17 duplicate samples of voidings submitted from the field, second aliquots of 38 previously analyzed urine samples were analyzed. The relative standard deviations for the analyses of these 55 duplicate samples averaged 13 (range

Table 2. Worker urinary aniline and *o*-toluidine concentrations.

Compound	Average urinary concentration ($\mu\text{g} \cdot \text{l}^{-1}$) ^a		Paired t-test ^b	
	Preshift	Postshift		
Aniline				
Unexposed group	2.7	3.8	0.01	(N = 31)
Exposed group	17.4	32.3	0.0001	(N = 46)
Unpaired t-test ^b	0.0001	0.0001		
o-Toluidine				
Unexposed group	1.1	2.7	0.0001	(N = 31)
Exposed group	18.5	103.7	0.0001	(N = 46)
Unpaired t-test ^b	0.0001	0.0001		

^a The levels of amine in the samples for which no peak was detected were calculated as the estimated limit of detection divided by the $\sqrt{2}$; there were 16 such samples for *o*-toluidine and four for aniline. In addition the *o*-toluidine levels reported for three samples were below the estimated limit of detection of $0.6 \mu\text{g} \cdot \text{l}^{-1}$, and the aniline levels reported for 19 samples were below the estimated limit of detection of $1.4 \mu\text{g} \cdot \text{l}^{-1}$.

^b The significance of the difference between the pre- and postshift samples of individuals was tested by the paired t-test (N = the number of subjects for which there were matched pre- and postshift urine specimens). The difference between the exposed and unexposed groups for both the pre- and postshift averages was tested by the unpaired t-test. In each case the data were log-transformed to achieve normality.

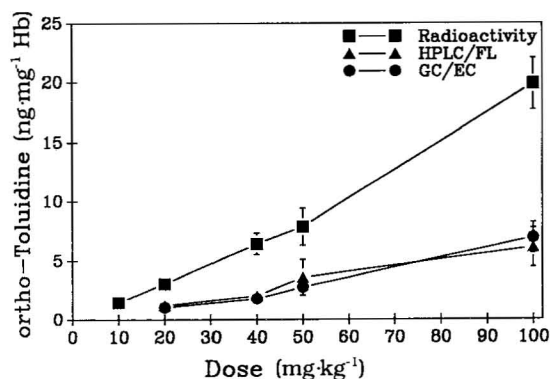


Figure 2. Effect of dose on hemoglobin binding in rats after the intraperitoneal administration of 10, 20, 40, 50, or $100 \text{ mg} \cdot \text{kg}^{-1}$ body weight [^{14}C]*o*-toluidine. *o*-Toluidine-hemoglobin adduct formation was determined either by high performance liquid chromatography (HPLC) with fluorescence (FL) or gas chromatography (GC) with electron capture (EC) detection or by ^{14}C levels on the assumption that all bound radioactivity was parent *o*-toluidine. Analysis by HPLC or GC was not performed for the $10 \text{ mg} \cdot \text{kg}^{-1}$ treatment group. The values represent means and standard deviations (N_5). (Hb = hemoglobin)

1–63)% for *o*-toluidine and 16 (range 1–65)% for aniline. Statistical analysis of these results revealed that the relative standard deviations did not vary significantly over the entire concentration range (0.8 – $530 \mu\text{g o-toluidine} \cdot \text{l}^{-1}$, <1.4 – $529 \mu\text{g aniline} \cdot \text{l}^{-1}$), a finding suggesting that the method was equally precise (or imprecise) throughout the range measured.

Biomarkers of internal dose. In vitro *o*-toluidine binding to either human or rat hemoglobin (8 – $9 \text{ ng} \cdot \text{mg}^{-1}$ hemoglobin) was similar under the conditions used. However, rat and human albumin did not show appreciable *o*-toluidine binding. The incubation conditions for albumin may not have been optimal, since

in vivo rat albumin binding has been reported (8). In addition, the possibility of increased or altered metabolism resulting from enzymatic induction was investigated. Hemoglobin incubated with microsomes prepared from phenobarbital-induced animals had over a 1.5-fold increase in *o*-toluidine binding (27.2 ng *o*-toluidine · mg⁻¹ hemoglobin versus 16.2 ng *o*-toluidine · mg⁻¹ hemoglobin with control microsomes).

The formation of *o*-toluidine-hemoglobin adduct was shown to be dose-dependent whether analyzed by bound radioactivity, HPLC, or GC analysis (figure 2). The results from the analysis of base hydrolyzable *o*-toluidine from hemoglobin by HPLC and GC were not statistically different. At the higher dose levels, a decreased percentage of bound *o*-toluidine could be hydrolyzed from the hemoglobin, a finding suggesting that other adducts which are not susceptible to base hydrolysis are formed.

Discussion

We have developed methods designed to measure aniline and *o*-toluidine in urine and *o*-toluidine adducted to hemoglobin and human albumin. All of these methods rely on the measurement of free amine(s) following base hydrolysis to convert acetylated metabolites (urine) or adducts back to the parent compound(s). Application of the urinary HPLC method to worker samples has been completed, while use of the new methods to detect adducts in worker blood specimens is currently in progress. An analysis of worker urine has clearly shown the uptake of both aniline and *o*-toluidine at the workplace. The data for *o*-toluidine are the most striking and indicate that the exposed workers are absorbing *o*-toluidine during the workshift, as the average concentration in the postshift samples compared with the preshift samples was sixfold higher. The average concentration of *o*-toluidine in the preshift samples of the exposed workers was 17 times higher than that of the unexposed workers, a finding suggesting a carry over of part of the *o*-toluidine dose from one workshift to the next. The roughly twofold increase in the average postshift levels of both amines in the unexposed workers over their average

preshift levels may be due to the overall higher pollutant levels in the workplace compared with off-work sites. Our new HPLC-fluorescence and GC-EC methods correlated well in the measurement of *o*-toluidine-hemoglobin adducts in in vivo experiments. The HPLC assay is less complex and is less susceptible to interference than the GC-EC procedure. On the other hand, the GC procedure does have better sensitivity than the HPLC-fluorescence method. Depending on exposure parameters, either method should be useful for detecting internal dose in human exposures. Although the analysis of the human samples is not complete, results of our in vivo and in vitro studies indicate that hemoglobin adducts are more useful than albumin adducts as a measure for *o*-toluidine exposure when analyzing for base-hydrolyzable adducts.

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