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

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Summary. Epidemiological evidence that occupational exposure to *o*-toluidine and aniline is associated with an increased risk of bladder cancer led to efforts to identify biomarkers of workplace exposures to these aromatic amines. For the determination of *o*-toluidine and aniline in worker urine specimens, a method using high performance liquid chromatography (HPLC) followed by electrochemical detection was developed. The limits of detection were 0.6 µg/l and 1.4 µg/l for *o*-toluidine and aniline, respectively. Recovery of *o*-toluidine and aniline from spiked urine averaged 86% and 93%, respectively, over a range of 4-100 µg/l. Reproducibility in the range 2-100 µg/l for analyses of split field samples was 13% (average RSD) for *o*-toluidine and 16% (average RSD) for aniline. Application of this method to pre- and post-shift samples collected from potentially exposed and unexposed workers indicated elevated concentrations of *o*-toluidine and aniline in urine from exposed workers. To develop methods for biomarkers of internal dose, *o*-toluidine binding to the blood proteins hemoglobin and albumin was investigated utilizing *in-vivo* (rodent) and *in-vitro* (hemoglobin and albumin) studies. Base-hydrolyzable protein adducts were analyzed by HPLC (fluorescence) and/or GC/electron capture (EC). The methods were compared for sample preparation requirements, selectivity and sensitivity. While the GC/EC method was more sensitive than HPLC, the presence of interfering peaks limited the utility of this approach. Results from these studies suggested that the HPLC method could be useful for determination of *o*-toluidine exposures in individuals acutely or chronically exposed to high levels.

Key Words: Aniline – Biological monitoring – *o*-Toluidine

Introduction

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 *o*-toluidine and aniline exposures, two of the principal chemicals used in the production processes [7,10]. NIOSH conducted a second study at this plant in 1990 to investigate worker exposures to *o*-toluidine and aniline by biological monitoring. The biological monitoring methods developed for the 1990 study and the results of analyses completed to date on the collected samples will be presented in this report.

A two-pronged effort was undertaken for determining exposures to these aromatic amines: 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-DNA adducts residing in inaccessible target tissue. The blood protein adducts generally are 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 (RBC) is 120 days, the level of adducted hemoglobin can reflect the average exposure over four months. Serum albumin has a half-life of 20-25 days in man. Assuming adduct half-life would parallel albumin half-life, after a single exposure adducted albumin could still be present up to 125 days, based upon first order kinetics and five half-lives for complete clearance [5].

Materials and 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 [1]. Rat data suggest that *o*-toluidine likewise is rapidly metabolized and excreted [2,8]. Thus biological monitoring using pre- and post-shift

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urine samples, to detect exposure during that workshift, seemed appropriate. While ring hydroxylation, followed by sulfate and glucuronide conjugation, is the major metabolic pathway for both aniline in humans [1] and *o*-toluidine in rats [2,8], small levels of the free amines are also present in urine, suggesting 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.* [4] quantified *o*-toluidine and aniline at the parts-per-billion level in urine from smokers and control subjects with no known occupational exposures. However, their methodology for sample workup 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, NaOH was added to a 4-ml aliquot of the urine until it was 4.7 *M*. The resulting solution was then heated at 80 ℃ for 2 hours. The neutral and basic compounds were extracted from this hydrolysate with butyl chloride, with the basic compounds subsequently back-extracted from the butyl chloride solution with 0.1 N HCl. An aliquot of the acidic extract was subjected to ion-interaction reversed-phase high performance liquid chromatography (HPLC) on a 30-cm x 6-mm column of Nova-pack C18 with coulometric electrochemical detection. The mobile phase was 60 mg/l sodium dodecyl sulfate and 53 *mM* PO_4^{3-} (pH 3.3) in 40% methanol/water (v:v).

Biomarkers of internal dose. The timing of sample collection for quantitation of protein adducts is less critical than for urinary biological monitoring. Lewalter and Korallus [6] have shown the presence of aniline-hemoglobin adducts in humans exposed to aniline in an industrial accident. Adduct levels peaked at 24 hours and were still detectable 7 days later. Stillwell, *et al.* [9] found an increase in the formation of *o*-toluidine-hemoglobin adducts for smokers compared to non-smokers (100 vs 34 pg *o*-toluidine/g hemoglobin) after base hydrolysis of the adducted hemoglobin. However, the technology used for quantitation of the parent amine (*o*-toluidine) by these investigators, GC/MS utilizing negative ion chemical ionization detection, was not available in our laboratory. 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 development of these analytical methods and to characterize *o*-toluidine-protein adduct formation. 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.

In-vitro studies. Rat or human hemoglobin or albumin was incubated with 130 nmol of [^{14}C]*o*-toluidine at 37 ℃ in a system consisting of rat liver microsomes and cofactor for 10, 20, 30, 45, or 60 min. Hemoglobin or albumin was isolated and ^{14}C levels were determined by liquid scintillation counting. Bound *o*-toluidine was quantified assuming all bound radioactivity represented parent *o*-toluidine. For induction studies, male Sprague-Dawley rats were pre-treated by intraperitoneal (ip) injection with 100 mg/kg body weight (bw) phenobarbital (PB) daily for 3 days, 80 mg/kg bw ß-naphthoflavone at 48 hours, or no treatment prior to sacrifice and preparation of microsomes.

In-vivo studies. The dose-effect for *o*-toluidine-hemoglobin adduct formation was tested in rats by administration of a single ip injection of [^{14}C]*o*-toludine at 10, 20, 40, 50, or 100 mg/kg bw with sacrifice at 4 hours. Blood was collected and hemoglobin isolated [3]. Protein-bound ^{14}C was determined by liquid scintillation counting. For analysis of adducted *o*-toluidine, hemoglobin was hydrolyzed with 1 *N* NaOH for 1 hr at 60 ℃ and extracted with ethyl acetate. HPLC analysis was performed using an automated precooling *o*-phthalaldehyde derivatization prior to methanol:water separation [3]. Separated components were detected by fluorescence (ex = 240 nm, em = 340 nm) and quantified using 4-ethylaniline as an internal standard. For GC analysis, the ethyl acetate extracts were back-extracted into 1 *N* HCl and concentrated. Samples were redissolved in benzene and derivatized with an excess of pentafluoropropionylimidazole. The derivatization procedure was carried out overnight at 60 ℃ and terminated by the addition of methanol. Samples were analyzed by temperature-programmed gas chromatography on a 30-m x 0.32-mm id column coated with a 1.5-µm thick film of Supelcowax™ 10. Separated components were detected by electron capture (EC) and quantified using 4-ethylaniline as an internal standard.

Field samples for analysis. Pre- and/or post-shift urine samples were obtained from 53 exposed and 36 nonexposed 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 ℃ until analysis. The 209 samples submitted from the field included 20 quality-control samples and duplicate 50-ml aliquots of 17 samples from workers.

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

Results

Urinary biological monitoring

Data for the recovery and precision for 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 no *o*-toluidine or aniline was lost from the samples during storage at -68℃ over a seven-month period. Using data from the analysis of 18 urine samples fortified to 1.4-15 µg/l plus 5 unfortified urine samples, the limit of detection (LOD) for *o*-toluidine was estimated to be 0.6 µg/l in urine. Similarly, the LOD for aniline was estimated to be 1.4 µg/l in urine.

Typical chromatograms are shown in Fig. 1. The results from 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 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 (*o*-toluidine, 0.8-530 µg/l; aniline, <1.4-529 µg/l) suggesting that the method was equally precise (or imprecise) throughout the range measured.

Table 1. Method recovery and precision using spiked urine samples

	Nominal Concentration (µg/l)[a]	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
o-Toluidine	4.2	10	101	32	64-183
o-Toluidine	20	10	93	17	75-125
o-Toluidine	102	10	86	14	65-98
N-Acetyl-*o*-toluidine	16 [c]	13	83	17	57-106

[a] 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.

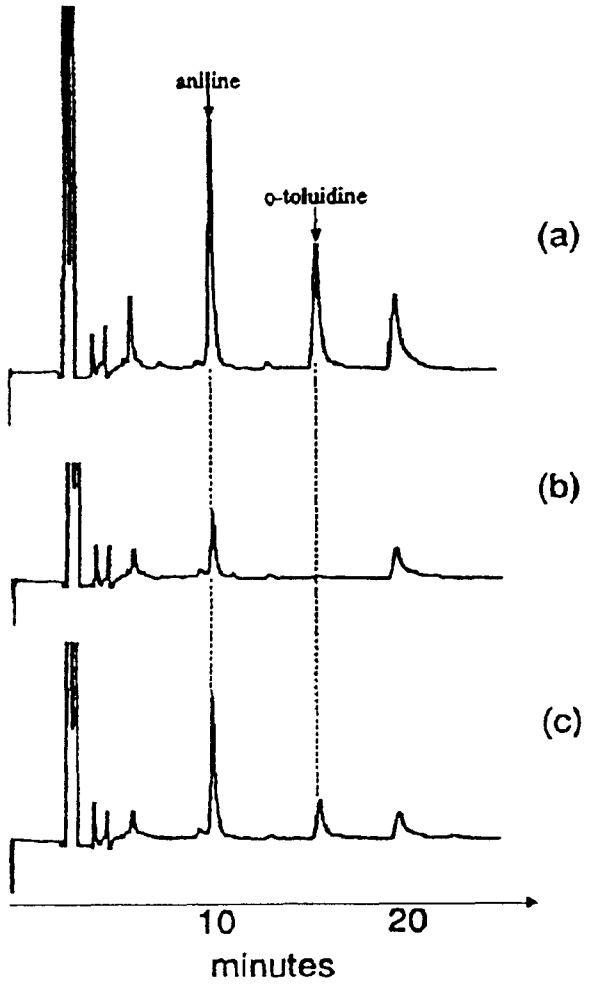

Fig. 1. Typical chromatograms: (a) field urine sample - 14.3 µg/l aniline and 11.8 µg/l *o*-toluidine found; (b) urine sample from a nonexposed individual - 4.0 µg/l aniline found; (c) same as (b) with - 4.0 µg/l aniline and 4.0 µg/l *o*-toluidine added.

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

	Mean urinary concentration (µg/l)[a] Pre-shift	Post-shift	Paired t-test[b]	
Aniline				
Unexposed	2.7 ± 2.4	3.8 ± 2.7	0.01	(N=31)
Exposed	17 ± 19	32 ± 26	0.001	(N=46)
Unpaired t-test[b]	0.0001	0.0001		
o-Toluidine				
Unexposed	1.1 ± 1.0	2.7 ± 1.4	0.0001	(N=31)
Exposed	18 ± 27	104 ± 111	0.0001	(N=46)
Unpaired t-test[b]	0.0001	0.0001		

[a] The arithmetic mean, followed by ± the arithmetic standard deviation, is given. The levels of amine in samples for which no peak was detected were calculated as the estimated LOD divided by the √2; there were 16 such samples for *o*-toluidine and 4 for aniline. Additionally, *o*-toluidine levels reported for 3 samples were below the estimated LOD of 0.6 µg/l and aniline levels reported for 19 samples were below the estimated limit of detection of 1.4 µg/l.

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

Biomarkers of internal dose

In-vitro o-toluidine binding to either human or rat hemoglobin (8-9 ng/mg hemoglobin) was similar at the conditions used; however, rat and human albumin did not show appreciable *o*-toluidine binding. Incubation conditions for albumin may not have been optimal, since *in-vivo* rat albumin binding has been reported [3]. Additionally, the possibility of increased or altered metabolism resulting from enzymatic induction was investigated. Hemoglobin incubated with microsomes prepared from PB-induced animals had over a 1.5-fold increase in *o*-toluidine binding (27.2 ng *o*-toluidine/mg hemoglobin versus 16.2 ng *o*-toluidine/mg 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 (Fig. 2). The results from the analyses of base hydrolyzable *o*-toluidine from hemoglobin by HPLC and GC were not statistically different ($P < 0.05$). At the higher dose levels, a decreased percentage of bound *o*-toluidine could be hydrolyzed from the hemoglobin, suggesting the formation of other adducts which are not susceptible to base hydrolysis.

Discussion

We have developed methods designed to measure aniline and

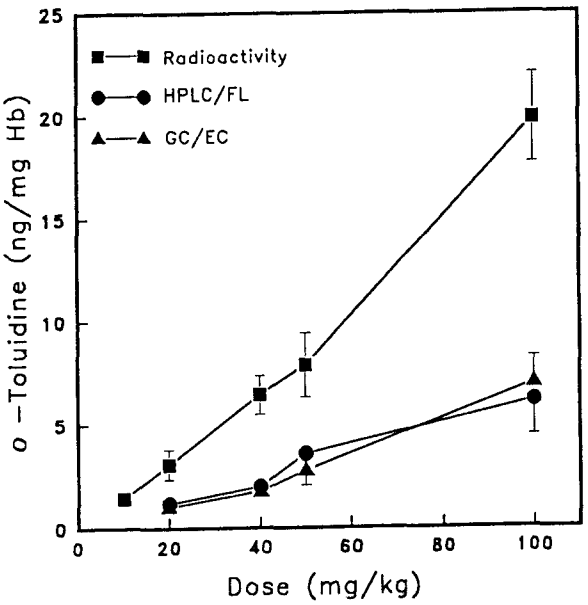

Fig. 2. The effect of dose on hemoglobin binding was investigated in rats after the ip administration of 10, 20, 40, 50, or 100 mg/kg body weight [^{14}C]*o*-toluidine. *o*-Toluidne-hemoglobin adduct formation was determined by either HPLC/fluorescence, GC/electron capture detection, or by ^{14}C levels assuming all bound radioactivity was parent *o*-toluidine. Analysis by HPLC or GC was not performed for the 10 mg/kg treatment group. Values represent means ± SD (N ≥ 5).

o-toluidine in urine and *o*-toluidine adducted to hemoglobin and human albumin. All 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 on-going. Analysis of worker urines has shown clearly the uptake of both aniline and *o*-toluidine at the workplace. The data for *o*-toluidine are most striking and indicate that the exposed workers are absorbing *o*-toluidine during the workshift, as the average concentration in the post-shift samples compared to the pre-shift samples is sixfold higher. The average concentration of *o*-toluidine in the pre-shift samples of the exposed workers was 17 times higher than that of the unexposed workers, suggesting carryover of a part of the *o*-toluidine dose from one work shift to the next. The roughly twofold increase in the average post-shift levels of both amines in the unexposed workers over their average pre-shift levels may be due to the overall higher pollutant levels in the workplace compared to off-work sites.

Our new HPLC-fluorescence and GC-EC methods have shown good correlation in the measurement of *o*-toluidine-hemoglobin adducts in *in-vivo* experiments. The HPLC assay is less complex and is less susceptible to interferences than the GC-EC procedure. On the other hand, the GC procedure does have better sensitivity than the HPLC fluorescence method; however, interfering substances limit its usefulness. Although analysis of human samples is not complete, it appears as the HPLC method would be useful for high-level exposures. Additionally, 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.

References

1. American Conference of Governmental Industrial Hygienists (1986) Aniline. Documentation of the Threshold Limit Values and Biological Exposure Indices. Cincinnati: ACGIH, BEI51-BEI53
2. Cheever KL, Richards DE, Plotnick HB (1980) Metabolism of *ortho*-, *meta*-, and *para*-toluidine in the adult male rat. Toxicol Appl Pharmacol 56:361-369
3. Cheever KL, DeBord DG, Swearengin TF, Booth-Jones AD (1992) *Ortho*-toluidine blood protein adducts: HPLC analysis with fluorescence detection after a single dose in the adult male rat. Fundam Appl Toxicol 18:522-531
4. El-Bayoumy K, Donahue JM, Hecht SS, Hoffmann D (1986) Identification and quantitative determination of aniline and toluidines in human urine. Cancer Res 46:6064-6067
5. Koch, DD, Miller, RC (1992) "Half-life" is erroneously used to describe the disappearance of erythrocytes and hemoglobin from the circulation. Clin Chem 38:1389
6. Lewalter J, Korallus U (1985) Blood protein conjugates and acetylation of aromatic amines. New findings on biological monitoring. Int Arch Occup Environ Health 56 :179-196
7. National Institute for Occupational Safety and Health (1989) Interim Report No. 1, HETA 88-159, Goodyear Tire and Rubber Company, Niagara Falls, NY, Cincinnati
8. Son OS, Everett DW, Fiala ES (1980) Metabolism of *o*-[*methyl*-^{14}C]toluidine in the F344 rat. Xenobiotica 10:457-468
9. Stillwell WG, Bryant MS, Wishnok JS (1987) GC/MS analysis of biologically important aromatic amines. Application to human dosimetry. Biomed Environ Mass Spectrom 14:221-227
10. Ward E, Carpenter A, Markowitz S, Roberts D, Halperin W (1991) Excess number of bladder cancers in workers exposed to *ortho*-toluidine and aniline. J Natl Cancer Inst 83:501-506