Identification and Quantitative Determination of Aniline and Toluidines in Human Urine

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ABSTRACT

Aniline and o-, m-, and p-toluidine, which are representative of aromatic amines in cigarette smoke, were identified and quantified in human urine. Smokers excreted 3.1 ± 2.6 µg/24 h of aniline and 6.3 ± 3.7 µg/24 h of o-toluidine (n = 16). Nonsmokers excreted 2.8 ± 2.5 µg/24 h of aniline and 4.1 ± 3.2 µg/24 h of o-toluidine (n = 12). Meta- and p-toluidine were detected in the urine of 2 of 11 smokers and 4 of 9 nonsmokers. The observed intra- and interindividual variations in the amounts of urinary aniline and o-toluidine were relatively large. The results of this study demonstrate for the first time that aniline and toluidines are present in human urine and suggest that sources other than cigarette smoke contribute significantly to their concentrations in urine.

INTRODUCTION

Industrial exposures to aromatic amines have been linked to urinary bladder cancer in man (1, 2). Cigarette smoking is also implicated in bladder cancer and aromatic amines are present in cigarette smoke (3, 4). However, data on levels of potentially carcinogenic aromatic amines in human urine are scarce. We are aware of only one study in which unspecified amounts of 2-naphthylamine and 2-amino-7-naphthol were tentatively identified in a smoker’s urine (5). In order to better assess the possible role of aromatic amines in bladder cancer etiology, it would be important to know the levels of these compounds and their metabolites in human urine. We have begun our investigations by analyzing human urine for aniline, o-, m-, and p-toluidine. These compounds were chosen because their concentrations in cigarette smoke exceed those of other aromatic amines and we expected that they would be readily detectable in smokers’ urine. However, as the results of this study demonstrate, the levels of these compounds in the urine of smokers and nonsmokers were similar, suggesting a major source for aromatic amine exposure other than cigarette smoke.

MATERIALS AND METHODS

Instruments. A Hewlett-Packard (Hewlett-Packard Co., Palo Alto, CA) Model 5830A gas chromatograph equipped with an HP-18850A terminal, and a 14N-labeled electron capture detector was used for detection and separation of pentafluoropropionamide derivatives of the aromatic amines. The separation was performed on a 50-m OV-101 (0.25 mm inside diameter; 0.25 µm film thickness) capillary column (Quadrex Corp., New Haven, CT). under the following conditions: the oven temperature was programmed from 90–200°C at 2°C/min with the injection port at 250°C. Helium was the carrier gas at a flow rate of 1 ml/min; 5% methane/argon was used as make-up gas. The split ratio was 25:1.

For selected ion monitoring of both the pentafluoropropionamide derivatives and the N-carbethoxy derivatives of aniline and the toluidines, we used an HP-5710A gas chromatograph combined with an HP-5980A mass spectrometer and an HP-5933A data system. Positive ion chemical ionization GLC-MS was performed at Rockefeller University, New York, NY, using an HP-5790A gas chromatograph and a 70-250 (VG Instruments, Stamford, CT) mass spectrometer with CH₄ as carrier gas. A 25-m 5% cross-linked phenylmethylsilicone capillary column was used (0.31 mm inside diameter; 0.52 µm film thickness). The oven temperature was programmed from 90–200°C at 2°C/min.

Chemicals. N-Acetyl-o-toluidine and N-carboxy-aniline (phenyl-urethane) were obtained from ICN Pharmaceuticals, Plainview, NY. Diethyl pyrocarbonate [(C₂H₅O₂C)₂O], triethylamine, aniline, o-, m-, and p-toluidine and m-ethylaniline were obtained from Aldrich Chemical Co., Milwaukee, WI. The amines were distilled before derivatization to the pentafluoropropionamides. 1- and 2-Naphthylamine and 2-, 3-, and 4-aminobiphenyl were obtained and purified as described (6) prior to derivatization. [methyl-14C]-o-Toluidine (7) was used as internal standard. N-carbethoxy-o-toluidine was synthesized as described (8); mp 44–45.5°C (lit. 44°C). Its purity was ascertained by thin layer chromatography and GLC: MS, m/e (relative intensity) 179 (M⁺, 99), 120 (M⁺, 54), 106 (100).

Derivatization and Hydrolysis Conditions. In preliminary studies, recoveries of ng to µg amounts of undervatized [methyl-14C]-o-toluidine from human urine were low (5–10%). Therefore, diethyl pyrocarbonate was added to the urine collection bottles as a derivatizing agent to prevent further reactions of the free amines. The reaction between amines and diethyl pyrocarbonate has been described (8).

Reaction conditions were investigated using 1 liter of distilled H₂O, to which 180–500 ng of [methyl-14C]-o-toluidine had been added. The pH, amounts of diethyl pyrocarbonate or ethanol, and reaction times were varied. The optimum conditions were found to be 50 ml of diethyl pyrocarbonate and 100 ml of ethanol/liter H₂O, and a minimum reaction time of 2.5 h at pH 6–6.5. The yield of [methyl-14C]-o-carbethoxy-o-toluidine was 70–80%. Conditions for hydrolysis of N-carbethoxy-o-toluidine to o-toluidine were also investigated. Conversions of 60–65% were obtained by heating with 15 ml 2 N NaOH under reflux and under N₂ for 2–2.5 h.

Analysis of Urine. Twenty-four-h urine collections were obtained from healthy male smokers and non-smokers, who were 25–45 years old. Fifty ml of diethyl pyrocarbonate was added to each urine collection bottle (brown; Nalgene; caps perforated to allow release of CO₂) along with 100 ml of absolute ethanol to inhibit bacterial growth and to increase the solubility of diethyl pyrocarbonate. After 24 h an appreciable amount of the diethyl pyrocarbonate remained in the urine collection bottle despite the fact that slow decomposition to CO₂ and ethanol had occurred. Immediately after collection, 180 ng of [methyl-14C]-o-toluidine (5 mCi/mmol; 1.9 x 10⁶ dpm) and an additional 20 ml of diethyl pyrocarbonate were added to the urine mixture. The mixture was stirred at room temperature for 2.5 h and concentrated by rotary evaporation under reduced pressure at 40°C. Either the entire urine mixture or a 200-ml aliquot was lyophilized. The residue was sonically dispersed in CHCl₃ (3 x 200 ml each) and filtered using a Buchner funnel and Whatman No. 1 filter paper. Recovery of internal standard was 70–80% at this point. The CHCl₃ extracts were combined, concentrated to dryness, and the residue was suspended in 15 ml of 2 N NaOH. The aqueous NaOH solution was heated under reflux with stirring under N₂ for 2.5 h to convert the N-carbethoxy amines back to the free amines. After sufficient refluxing, the solution was saturated with NaCl and extracted twice with CHCl₃ (3 x 40 ml each). The extracts were combined, concentrated to dryness, and resuspended in 1 ml of benzene.

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2 To whom requests for reprints should be addressed.

The abbreviations used are: GLC, gas chromatography; MS, mass spectrometry.
The benzene solution was placed in a 5-ml Reactitherm vial (Pierce Chemical Co., Rockford, IL) containing a small stirring magnet. To the vial was added 3 ml of 0.05 m triethylamine in benzene and 1 ml of pentafluoropropionic anhydride. The mixture was heated with stirring at 55°C for 20 min, allowed to cool, and then transferred to a 25-ml Reacti-Flask. Ten ml of distilled H2O followed by 10 ml of 5% NH4OH were added to the mixture. Under these conditions, derivatization to the pentafluoropropionamides went essentially to completion. Recovery of internal standard before and after derivatization was essentially identical and was approximately 30–50%. Two-µl aliquots of the benzene layer were injected onto the GLC column. Amounts of aromatic amines in each aliquot were determined by comparison to calibration curves constructed with standards. Values were corrected for losses during work-up as determined by scintillation counting.

Retention times (min) were typically as follows: for the pentafluoropropionamides of aniline (19.4); o-toluidine (23.5); m-toluidine (26.9); p-toluidine (28.1); m-ethylamine (34.9); 1-naphthylamine (64.7); 2-naphthylamine (71.6); 2-aminobiphenyl (67.8); 3-aminobiphenyl (89.4); 4-aminobiphenyl (92.6). Student’s t test was used for statistical analysis.

RESULTS

The analytical scheme is outlined in Fig. 1. Aromatic amines in urine were derivatized to the corresponding N-carbethoxyamines by reaction with diethyl pyrocarbonate. [methyl-¹⁴C]p-Toluidine was added as internal standard. After lyophilization and extraction, the CHCl₃-soluble N-carbethoxyamines were hydrolyzed to the free amines. These were converted to the corresponding pentafluoropropionamides which were analyzed by capillary GLC with electron capture detection. The detection limit for standards was approximately 1 pg/injection. The detection limit for aniline and toluidines in urine was approximately 50–100 ng/24 h. Recovery of internal standard ranged from 30–50%.

N-Acetyl-o-toluidine was also hydrolyzed to o-toluidine under the conditions used for conversion of N-carbethoxy-o-toluidine to o-toluidine. Thus, the method does not distinguish between aromatic amines and the corresponding acetamides.

Fig. 2 is a typical gas chromatogram of the derivatized aromatic amine fraction from a smoker’s urine. Peaks with retention times corresponding to the pentafluoropropionamide derivatives of aniline, o-, m-, and p-toluidine were observed as indicated. This fraction was analyzed by GLC-MS with selected ion monitoring. The three major peaks in the MS of the pentafluoropropionamide derivatives of aniline and o-toluidine are the molecular ions and the peaks due to loss of C₅F₅ and loss of NHCO₂F₅. These are m/e 239, 120, and 77 for aniline and m/e 253, 134, and 91 for o-toluidine. Selected ion monitoring demonstrated that these three ions were present only at the correct GLC retention times of aniline and o-toluidine. Confirmation of the identities of aniline, o-, m-, and p-toluidine was obtained from their positive ion chemical ionization MS. Representative MS are shown in Fig. 3. Similar spectra were obtained for m- and p-toluidine. Although some samples showed peaks at the retention time of 2-naphthylamine, its presence could not be confirmed by GLC-MS.

A typical gas chromatogram of the derivatized aromatic amine fraction from a nonsmoker’s urine is shown in Fig. 4. The identities of the indicated peaks were confirmed by GLC-MS. The aromatic amine peaks were not detected in an H₂O blank.

Table 1 summarizes the results of the analyses of aniline and toluidines in human urine. Levels of aniline and o-toluidine in the urine of subjects 1–3 differed greatly on days 1–3 and
ANILINE AND TOLUIDINES IN HUMAN URINE

Fig. 4. Gas chromatogram of the derivatized aromatic amine fraction from non-smokers' urine. EC, electron capture.

Table 1 Aniline and toluidines in human urine

Twenty-four-h urine samples were collected and analyzed as illustrated in Fig. 1 and described in "Materials and Methods." All values were corrected based on the recovery of the internal standard.

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<th>Subject</th>
<th>No. of cigarettes smoked</th>
<th>Aniline (µg/24 h)</th>
<th>o-Toluidine (µg/24 h)</th>
<th>m-Toluidine (µg/24 h)</th>
<th>p-Toluidine (µg/24 h)</th>
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ND = not detected; NQ = not quantified due to interfering peak.

*Sample divided into 3 portions which were analyzed individually; aniline, 2.0 ± 0.6 (SD); o-toluidine, 4.1 ± 0.3. In another experiment the sample was divided into 4 portions which were analyzed individually; aniline, 1.5 ± 0.4; o-toluidine, 3.0 ± 0.3.

showed no apparent relationship to the number of cigarettes smoked. Large intra- and interindividual variations in the amounts of urinary aniline and o-toluidine were observed. m- and p-toluidine were less frequently detected than were aniline and o-toluidine.

Fig. 5 compares all data for aniline and o-toluidine in smokers and non-smokers. Their mean values were not significantly different in smokers and non-smokers.

DISCUSSION

The results of this study demonstrate for the first time that aniline and toluidines are present in human urine. Several other aromatic amines such as 1- and 2-naphthylamine and 2-, 3-, and 4-aminobiphenyl were not detected. However, no attempt was made to analyze for putative metabolites of the aromatic amines so the present results do not exclude human uptake of these compounds. Indeed, recent studies have demonstrated the presence in human hemoglobin of adducts formed from the toluidines and 4-aminobiphenyl (9). It will be important to establish the relationship between urinary levels of aromatic amines or their metabolites and the amounts of the corresponding hemoglobin adducts.

The concentrations of aniline and o-toluidine in smokers' urine were not significantly different from those in non-smokers' urine. A complete assessment of exposure to these compounds must await development of methods to measure their metabolites. Nevertheless, the results do suggest that exposure to aniline and o-toluidine or their precursors can occur independent of cigarette smoking. What are the origins of aniline and o-toluidine in human urine? High levels of these aromatic amines are present in sidestream smoke of cigarettes (4). However, none of our non-smokers were extensively exposed to environmental tobacco smoke. Aniline and o-toluidine are important industrial chemicals (10, 11). Aniline is an intermediate in the production of a variety of polyurethane products, in the preparation of compounds used in rubber manufacturing, in the synthesis of dyes, and in the production of pharmaceuticals. o-Toluidine is used mainly in the manufacture of dyes. Levels of residual aniline and o-toluidine in any of these products have not been reported. Such products could be a source of direct exposure to aniline or o-toluidine or to their metabolic precursors such as azo dyes. Aniline and o-toluidine have been detected in surface water samples, probably due to their extensive industrial production (12). They do not seem to have been

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detected in industrial air pollution, although their presence might be expected because they have been found in various vegetables such as cauliflower, carrots, and celery (12). Aniline and o-toluidine have been identified in the volatile aroma compounds of black tea (13). Limited information is available on levels of aniline and toluidines in cooked foods but aromatic amines are commonly detected in pyrolysates of amino acids and proteins and their presence in broiled foods would be expected (14, 15). Endogenous formation of aniline or toluidines are commonly detected in pyrolysates of amino acids pounds of black tea (13). Limited information is available on the diet may be a major source of urinary aniline and toluidines and are currently carrying out controlled diet studies to test this hypothesis.

O-toluidine induces a variety of tumor types in rats including papilloma and transitional cell carcinoma of the urinary bladder (10, 16–18); m- and p-toluidine are inactive or weakly carcinogenic in rats and mice (17, 18). High levels of dietary aniline cause tumors of the spleen in rats (11). According to the International Agency for Research on Cancer, there is sufficient evidence for the carcinogenicity of o-toluidine in experimental animals, but limited evidence for aniline (10, 11). Further studies are required to determine whether the presence of o-toluidine in human urine is related in any way to cancer incidence.

REFERENCES

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