Estimation of N-hydroxy-o-toluidine, a urinary metabolite of o-toluidine and o-nitrosotoluene, by high performance liquid chromatography with electrochemical detection

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Abstract

High-performance liquid chromatography with electrochemical detection, a highly sensitive and specific method, was used to determine N-hydroxy-o-toluidine and o-toluidine in the urines of male F344 rats after the administration of 0.82 mmol/kg of o-toluidine or o-nitrosotoluene. In a six hour period, 0.11% of the dose was excreted by o-toluidine-treated rats as N-hydroxy-o-toluidine and 3.61% as o-toluidine. In the same period, 0.48% of the dose was excreted by o-nitrosotoluene treated rats as N-hydroxy-o-toluidine and 0.38% as o-toluidine. The urinary excretion of the N-hydroxy-o-toluidine metabolite may play a role in the carcinogenicity of o-nitrosotoluene and o-toluidine toward the rat bladder.

Introduction

There is now strong experimental support for the concept that the induction of urinary bladder cancer in various species by dual-ring aromatic amines such as 4-aminobiphenyl (1,2), 2-naphthylamine (2,3) and 3,2'-dimethyl-4-aminobiphenyl (4), is linked with the excretion of the respective N-hydroxy-N-glucuronide metabolites in the urine (5–10). Such N-glucuronides are easily hydrolyzed, especially under mildly acidic conditions to the direct acting mutagenic (11–13) and carcinogenic (14,15) N-hydroxy aglycones. The latter are readily absorbed by the bladder epithelium (16) and have been shown to react with DNA without further enzymatic activation (17,18).

Single-ring aromatic amines such as o-toluidine (19,20), o-anisidine (21) and m- and p-cresidines (22,23) have also been shown to induce bladder tumors in rodents. In contrast to the dual-ring aromatic amines, however, the proximate and ultimate carcinogenic forms of the single-ring compounds have not as yet been identified.

In the case of o-toluidine, the simplest carcinogenic aromatic amine, in vivo metabolism studies have shown that o-nitrosotoluene and 2,2'-dimethoxybenzene are present in the urines of F344 rats following the s.c. administration of the parent amine (24). Such compounds could represent metabolites resulting from the oxidation of the primary metabolite, N-hydroxy-o-toluidine, during isolation procedures. Evidence that an N-oxidized form of o-toluidine is responsible for bladder carcinogenicity was provided by bioassays in which o-nitrosotoluene was shown to be several fold more carcinogenic for the bladder in F344 rats than o-toluidine (25).

In the present communication we describe the use of a highly sensitive and selective h.p.l.c. electrochemical detector method for the determination of N-hydroxy-o-toluidine in the urine of F344 rats. With this method we demonstrate that o-toluidine is N-oxidized to N-hydroxy-o-toluidine and that o-nitrosotoluene is reduced to N-hydroxy-o-toluidine as well as o-toluidine in vivo.

Materials and Methods

Chemicals

o-Toluidine, o-nitrosotoluene and 4-amino-m-cresol were obtained from Aldrich Chemical Co., Milwaukee, WI. Prior to administration to rats, o-toluidine was purified by vacuum distillation and o-nitrosotoluene was recrystallized from benzene-hexane. N-hydroxy-o-toluidine was prepared by the reduction of o-nitrosotoluene (Aldrich) with Zn/NH4Cl. N-Acetyl-N-hydroxy-o-toluidine was prepared by treating N-hydroxy-o-toluidine with acetic anhydride in ethyl acetate under N2. The synthesis and purification of these compounds followed closely the procedures described by Hecht et al. (12). Purities of the recrystallized N-hydroxy compounds were determined by h.p.l.c. and mass spectrometry. N-Acetyl-o-toluidine was obtained from ICN Pharmaceuticals, Inc., Plainview, NY. All other chemicals were either reagent or h.p.l.c. grade. Distilled deionized H2O (18.3 megohm resistivity) was used in all phases of these experiments.

Animals

Male F344 rats (Charles River Breeding Laboratories, Kingston, NY), 230–260 g body weight, were maintained on NIH-07 diet and water ad libitum. For metabolism studies, rats were injected s.c. with 0.82 mmol/kg of either o-toluidine or o-nitrosotoluene as corn oil solutions, placed in stainless steel metabolism cages and urine was collected over a period of 6 h in containers maintained at 70°C. All urines were analyzed within 4 h of collection.

Analytical

A DuPont Zorbax C8 column (4.6 x 250 mm) preceded by a 4.8 x 60 mm guard column containing Co/Pd ODS packing (Whatman) was eluted at 1.0 ml/min with 50:50 (v/v) methanol-H2O, 8 mM in potassium phosphate buffer pH 7.4, using a Waters Model 6000A h.p.l.c. pump with a Model U6K injector. The outlet of the column was connected to a Metrohm Modell EA1096 Kel-F electrochemical detector flow cell with glassy carbon indicating and working electrodes and a Ag/AgCl reference electrode. A Metrohm Model 641 VA-detector was used to control the cell potential and to measure the resulting current. The electrochemical detector flow cell in turn was connected to a Schoeffel Model SF 770 Spectroflow monitor for determination of u.v. absorption at 254 nm.

For analysis of N-hydroxy-o-toluidine and o-toluidine, urines were thawed at 0°C and 0.05 ml aliquots were diluted to 0.15 ml by the addition of ice-cold 95:5 (v/v) methanol-H2O, 8 mM in potassium phosphate buffer, pH 7.4. Following centrifugation at 0°C, 10 μl aliquots of the clear supernatants were immediately submitted to h.p.l.c.

Results

Electrochemical detector response

Previous work in this laboratory has shown that 4-amino-m-cresol (2-methyl-4-hydroxyaniline), N-acetyl-o-toluidine, o-toluidine and o-nitrosotoluene are found in the urines of F344 rats following the administration of o-toluidine (24). For reference purposes, the h.p.l.c. elution volumes of these compounds and of N-hydroxy-o-toluidine, determined by their u.v. absorption at 254 nm, are shown in Figure 1. While N-hydroxy-o-toluidine is well resolved from o-toluidine, N-oxidized to N-hydroxy-o-toluidine and that o-nitrosotoluene is reduced to N-hydroxy-o-toluidine as well as o-toluidine in vivo.

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Fig. 1. H.p.l.c. elution volumes of several non-acid-conjugated o-toluidine metabolites (24) (cf. Materials and Methods). N-Hydroxy-o-toluidine co-elutes with N-acetyl-o-toluidine. X is an impurity present in the 4-amino-m-cresol standard.

If labelled o-toluidine were administered) using this or similar h.p.l.c. systems could be subject to error because of possible co-elution with other o-toluidine metabolites. In this respect, the advantage of the electrochemical method for selective detection of the N-hydroxy metabolite is illustrated in Figure 2. The data summarized in this figure were obtained by submitting 5.7 nmol of either N-hydroxy-o-toluidine, o-toluidine, N-acetyl-o-toluidine or N-hydroxy-N-acetyl-o-toluidine (a possible though as yet unconfirmed o-toluidine metabolite) to h.p.l.c. with the potential of the electrochemical detector set at values ranging from 0 to +1200 mV versus the Ag/AgCl electrode. In the case of N-hydroxy-o-toluidine, the threshold potential for the appearance of detectable current peaks due to oxidation reactions at the glassy carbon electrode was approximately +50 mV. From +600 to +1000 mV, a plateau in the electrochemical detector response was observed. For these determinations, as well as for all others in which N-hydroxy-o-toluidine was used, the compound was weighed, dissolved in N2-flushed methanol-

Fig. 2. Electrochemical detector response to N-hydroxy-o-toluidine, o-toluidine, N-hydroxy-N-acetyl-o-toluidine and N-acetyl-o-toluidine (5.7 nmol each) in the h.p.l.c. effluent as a function of applied potential (versus the Ag/AgCl reference electrode).

In the case of o-toluidine, no response was obtained at potentials at or below +700 mV; at potentials more positive than 800 mV, a steep increase in detector response occurred. For either N-acetyl-o-toluidine or N-hydroxy-N-acetyl-o-toluidine, no response was obtained at potentials lower than approximately +1000 mV. o-Nitrosotoluene gave no response within the range of 0 to +1200 mV under these conditions. This suggests that the amine and N-hydroxy functions of o-toluidine and N-hydroxy-o-toluidine, respectively, are oxidized to the nitroso stage only; further oxidation to the nitro stage apparently does not occur at the glassy carbon electrode under these conditions.

The relationships between the amounts of o-toluidine (electrode potential +900 mV) or N-hydroxy-o-toluidine (+600 mV) submitted to h.p.l.c. and peak current detector response were linear up to at least 500 ng for each compound. The limits of detectability (peak height ~2x baseline noise) were 0.005 nmol for o-toluidine and 0.04 nmol for N-hydroxy-o-toluidine.

N-Hydroxy-o-toluidine in urine of rats treated with o-toluidine

Typical profiles of urine from a rat treated with o-toluidine using electrochemical detector potentials of +900 and +600 mV are shown in Figure 3. At +900 mV, a large interfering peak, most likely due to phenolic metabolites, their conjugates (24) and unidentified compounds present in urine, eluted between 2 and 6 ml. A shoulder on this peak, at 6.5 ml, corresponding to the elution volume N-hydroxy-o-toluidine was observed. This was followed by a large peak due to o-toluidine (8.0 ml). While o-toluidine was easily detected by its u.v. absorption at 254 nm, in the case of the N-hydroxy-o-toluidine, no u.v. absorption peak was discernible due to the presence of interfering components and the minute quantity of this metabolite.

At the electrode potential of +600 mV (lower tracing in Figure 3), baseline resolution of the peak corresponding to N-hydroxy-o-toluidine was obtainable due to greatly reduced interference from early eluting urinary components and from the complete disappearance of the o-toluidine peak. Determinations made on control rat urines spiked with N-hydroxy-o-toluidine showed that recovery ranged from 98 to 102%.

N-Hydroxy-o-toluidine in urine of rats treated with o-nitroso-toluene

The h.p.l.c.-electrochemical detector analysis of urines obtained from male rats treated with 0.82 mmol/kg body weight of o-nitrosotoluene, s.c., was carried out in the same way as above. Typical elution profiles so obtained are shown
in Figure 4. At the detector potential of +900 mV, peaks corresponding to N-hydroxy-o-toluidine as well as o-toluidine were observed, including in vivo reduction of o-nitrosotoluene to both these species. The amounts of o-toluidine and N-hydroxy-o-toluidine excreted in the 6 h period after the administration of o-toluidine or o-nitrosotoluene are shown in Table I.

The greater urinary levels of N-hydroxy-o-toluidine after the administration of o-nitrosotoluene permitted additional verification of the N-hydroxy metabolite as follows. A portion of the 0—6 h urine obtained from o-nitrosotoluene treated rats was extracted with ethyl acetate and the ethyl acetate extract was taken to dryness under vacuum using a Savant Speed-Vac centrifugal evaporator. The residue was redissolved in a small volume of methanol-\( \text{H}_2\text{O} \) (95:5), 8 mM in potassium phosphate buffer, pH 7.4, at 0°C. A 10μl aliquot of this solution was then submitted to h.p.l.c. without the application of an oxidizing potential to the electrochemical detector (detector on 'standby'), and the effluent between 6.0 and 7.0 ml elution volume, i.e., corresponding to the elution volume of N-hydroxy-o-toluidine, was collected on ice. A 10 μl portion of the collected fraction was then immediately resubmitted to h.p.l.c. with electrochemical detection at a potential of +900 mV. As shown in the lower tracing of Figure 5, this sample contained only N-hydroxy-o-toluidine. To 1 ml of the remaining portion of the collected sample was then added 50 mg of Zn dust and 20 μl of glacial acetic acid. This mixture was kept at 25°C with stirring for 30 min. Following clarification by centrifugation, an aliquot of the supernatant was submitted to h.p.l.c. with electrochemical detection at +900 mV. The resulting profile (upper tracing in Figure 5) showed the presence of o-toluidine, resulting from the reduction of the N-hydroxy metabolite with Zn/acetic acid. Since no other urinary component (except o-nitrosotoluene which elutes at ~33 ml) can yield o-toluidine under the mild reducing conditions used, this provides additional proof that the peak eluting at 6.5 ml indeed represents N-hydroxy-o-toluidine.

### Discussion

The h.p.l.c.-electrochemical method for the detection of N-hydroxy-o-toluidine used in this work combines the advantages of the high resolution inherent in h.p.l.c. with the selectivity and high sensitivity of the electrochemical detector. Previously, similar methods have been reported for the determination of aromatic amines (26), aminophenols (27) and of derivatized N-hydroxyarylamines (28,29). The direct assimi-
tion of aliphatic and aromatic hydroxyamines in aqueous solution by amperometric methods has also been reported (30). The method presented here does not involve derivatization and requires only minimal sample work-up. Recently, Frederick et al. (18,31) have described an h.p.l.c. method for the selective estimation of N-hydroxy and ring-hydroxy aromatic amine metabolites which also does not require derivatization, and which is based on the post-column reduction of Fe³⁺ to Fe²⁺ by these compounds. The Fe²⁺ is then detected as the colored complex it forms with bathophenanthroline. The detection limit of this method was reported as 0.6 nmol of N-hydroxyl-1-naphthylamine or N-hydroxy-2-naphthylamine. The detection limit of the electrochemical method described here is 0.04 nmol for N-hydroxy-o-toluidine.

The amounts of N-hydroxy-o-toluidine found in the 0—6 h urines of rats treated with 0.82 nmol/kg of o-toluidine ranged from 0.02 to 0.18 (x = 0.11)% of the dose. In the case of rats treated with equimolar amounts of o-nitrosotoluene, the urinary excretion of the N-hydroxy metabolite ranged from 0.23 to 0.67 (x = 0.48)% of the dose. Because of the possible lability of the compound during storage in the bladder and some degree of absorption by the bladder epithelium (16), it is probable that the amounts of N-hydroxy-o-toluidine reaching the bladder are higher than those we actually detected in the voided urine. It is also possible that, as is the case with several dual-ring aromatic amines (5—10), a portion of the excreted N-hydroxy metabolite may be present as the N-glucuronide conjugate. The latter was not looked for in the present investigations.

It is tempting to relate the presence of N-hydroxy-o-toluidine in rat urine after the administration of both o-toluidine and o-nitrosotoluene with the documented carcinogenicity of the parent compounds toward the rat urinary bladder (19,20,25). While non-enzymatic activation of N-hydroxyarylamines to reactive electrophilic species occurs under mildly acidic conditions, e.g., pH 5—6, as in the case of dog or human urine (6,8,9,17), binding of N-hydroxy-2-aminofluorene to DNA has been observed even at pH 7.5 (18). The reactivity of N-hydroxy-o-toluidine with model and biological nucleophiles under conditions approximating those within the rat bladder (pH 6.6—6.7) is currently being investigated in this laboratory.

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