Identification of Sulfate and Glucuronic Acid Conjugates of the 5-Hydroxy Derivative as Major Metabolites of 2-Amino-3-methylimidazo[4,5-f]quinoline in Rats

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ABSTRACT

New metabolites of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), a potent mutagen and carcinogen formed during cooking of meat or fish, have been identified and quantitated in the urine and bile of rats. Administration was either by a pulse gavage dose of 40 mg/kg [2-14C]IQ or by dietary intake of 300 ppm IQ for 6 weeks. The metabolites were isolated by high-performance liquid chromatography and quantitated by radioactivity. They were then characterized by their resistance or sensitivity to hydrolytic enzymes or acid hydrolysis, by nuclear magnetic resonance and mass spectrometry, or coinjection with a synthetic sample. A minor metabolite was the IQ N-glucuronide. A major metabolite was formed by hydroxylation of IQ at the 5-position; it was present in urine and bile and was conjugated as the glucuronide or sulfate ester, which together accounted for about 40% of urinary or biliary metabolites. The unconjugated compound partially adsorbs onto the high-performance liquid chromatographic columns used. The amounts of 5-OH-IQ present as conjugates in urine or bile were similar, irrespective of mode of administration. Thus, hydroxylation of IQ on carbon 5 followed by type II conjugation reactions yields quantitatively important metabolic products.

INTRODUCTION

Heterocyclic amines typified by having a 2-amino-3-methyl or similar α-methylamine arrangement in the molecular formula represent a new class of carcinogens with wide environmental human contact since this type of chemical is found in broiled or fried meats and fish (1–4). It is important to understand the mode of action of these heterocyclic amines to define their potential role in human cancer causation.

Previous reports described the methods evolved in this laboratory to permit the HPLC resolution of metabolites isolated from urine, bile, and feces of rats given IQ, a typical mutagenic/carcinogenic heterocyclic amine (5, 6). Urine and bile contained three major and some minor polar metabolites. One of the major metabolites was the sulfamate of IQ found in urine, bile, and feces (6, 7).

This paper describes the identification and quantitation of three polar metabolites of IQ, one minor, two major, obtained in the HPLC analysis of urinary and biliary metabolites. The major metabolites were present as conjugates in urine and bile, but were absent from feces, suggesting that they are conjugates capable of being split by hydrolytic enzymes in intestinal microflora. It will be shown that these metabolites are the sulfate ester and the glucuronic acid conjugate of 5-OH-IQ, previously referred to as metabolite M-2, and M-1, respectively (6). The minor metabolite was IQ N-glucuronide, m-1.

MATERIALS AND METHODS

Chemicals and Reagents. [2-14C]IQ (10 mCi/mmOL) and unlabeled IQ were purchased from Toronto Research Chemicals, Toronto, Canada (Dr. David Dime). [5-3H]IQ (2.43 Ci/mmOL) was obtained from Chem-syn Science Laboratories, Lenexa, KS, the NCI Chemical Carcinogen Repository Laboratory for the Division of Cancer Etiology, National Cancer Institute (Dr. D. Longfellow). Purity of all IQ samples was checked by HPLC and found to be greater than 99% (data not shown). β-Glucuronidase, Bacterial Type VIII, and sulfatase type VIII were purchased from Sigma Chemical Company, St. Louis, MO. All other chemicals were of analytical grade or higher.

Preparation of IQ N-Glucuronide. IQ (10 µmol) was incubated for 60 min at 37°C in 1 ml 100 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂, 100 µM phosphatidylocholine (Sigma), 5 mM uridine 5'-diphosphoglucuronic acid ammonium sodium salt (Sigma) and 40 mg (protein) of solubilized rat liver S-9 fraction prepared accordingly to Green and Tephly (8). The reaction was terminated by adding 20 ml ethanol. The precipitated protein was removed by filtration and the remaining solution was evaporated in vacuo, and reconstituted in 2 ml H₂O. Upon passage through a Sep-Pak C-18 cartridge IQ was retained, and the N-glucuronide was eluted with a 1% methanol/H₂O solution. The N-glucuronide was purified by HPLC (see analysis of metabolites) and isolated in a yield of 1.2%. On NMR spectroscopy this compound displayed a chemical downfield shift typically seen with NH compounds, but, as expected, did not show the shift associated with NH in IQ (Table 1). The mass spectrum, obtained in the presence of ammonium chloride to assist in the volatilization of the polar compound, had the following correct values: M + NH₄, 390; M - glu + H⁺, 198; glu-COOH + H⁺, 132. The biosynthetic N-glucuronide was used for verification of the identification of metabolite m-1.

Treatment of Animals. Adult male Fischer F344 rats were purchased from Charles River Laboratories, Kingston, NY. After a quarantine period and health testing, they were transferred to the specialized metabolism room in the Research Animal Facility of this Institute, accredited by the AAALAC. The diet was a commercial NIH-07 formulation from Zeigler Brothers, Gardners, PA. Drinking water was freely available. The rats were housed in stainless steel metabolism cages (Acme Research Products, Cincinnati, OH) that allow for separation from the experimenter. The rats were acclimatized in the metabolism cages for 3 days and transferred to identical clean cages after IQ dosing. Doses of 40 mg/kg body weight of [2-14C]IQ (10 µCi/rat) dissolved in 1 ml of 0.1 N HCl and adjusted to pH 3 were given by gavage. Each rat also received 5 ml sterile saline at the time of dosing to yield more urine. Administration of IQ was performed twice, with a 48-h period between dosings to increase the amount of available metabolites for study. In the same way, [5-3H]IQ was administered to four rats.

Administration by Gavage. The rats (body weights about 300 g) were acclimated in the metabolism cages for 3 days and transferred to identical clean cages after IQ dosing. Doses of 40 mg/kg body weight of [2-14C]IQ (10 µCi/rat) dissolved in 1 ml of 0.1 N HCl and adjusted to pH 3 were given by gavage. Each rat also received 5 ml sterile saline at the time of dosing to yield more urine. Administration of IQ was performed twice, with a 48-h period between dosings to increase the amount of available metabolites for study. In the same way, [5-3H]IQ was administered to four rats.

Uranes were collected in test tubes cooled by dry ice and methanol during the two successive 24-h periods following each dosing of IQ. Urine samples for each collection period were thawed and centrifuged (3000 rpm for 15 min) to remove any particulate matter, then stored at -20°C until assayed.

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3 The abbreviations used are: HPLC, high-performance liquid chromatography; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; m-1, IQ-N-glucuronide; 5-OH-IQ, 2-amino-3-methyl-5-hydroxymidazo[4,5-f]quinoline; M-1, 5-OH-IQ glucuronide; M-2, 5-OH-IQ sulfate ester; M-3, IQ-N-sulfamate or N-(3-methylimidazo[4,5-f]quinolin-2-yl) sulfamic acid; NMR, nuclear magnetic resonance, NOE, nuclear Overhauser effect; DMSO-d₆, deuterated dimethyl sulfoxide.

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was dissolved in 1:1 potassium phosphate buffer (20 mm, pH 4.72):methanol, filtered, and stored at —20°C. The radioactivity was followed by evaporating in a rotary flask to dryness in vacuo. The resulting residue was taken up with an equal volume of water. The metabolites were extracted by refluxing each sample twice with 50 ml of double-distilled water for 45 min. The volume of water was used as the running buffer.

Analytical HPLC was used to insure a purity greater than 95%, required for instrumental analysis. In the analytical HPLC procedures, an analytical Whatman Partisil-5 ODS-3 (250 mm x 4.6 mm) was used. The flow rate was 2.5 ml/min. After initial purification of metabolites by preparative HPLC, analytical HPLC was used to insure a purity greater than 95%, required for instrumental analysis. In the analytical HPLC procedures, an analytical Whatman Partisil-5 ODS-3 (250 mm x 4.6 mm) was used. The gradient was identical to the aforementioned one, but with a flow rate of 1 ml/min. As a final clean-up, each isolated metabolite was rechromatographed with the identical gradient, but at a 0.2% acetic acid solution was used as the running buffer.

Excretion of metabolites in Urine and Bile. The overall excretion of radioactivity in urine and feces after administration of [2-14C]IQ and [5-3H]IQ has been described (6). In the HPLC separation of urine and bile three major polar metabolites, designated M-1, M-2, and M-3, and a minor polar metabolite (m-1) were obtained (Fig. 1). M-3 was shown to be IQ-sulfate, as determined by NMR (Table 1). Dilute acid hydrolysis gave a quantitative conversion to IQ only. Turesky et al. (7) also observed this metabolite, with identical properties.

This report deals with the characterization of m-1, M-1, and M-2. These metabolites are present in urine and bile, and not in feces, suggesting they can be cleaved by the hydrolytic enzymes of the intestinal bacterial flora.

Identification of m-1 as IQ β-Glucuronide. Resolution by HPLC of urine from rats given [2-14C]IQ yields a small peak at a mobility of 51 min, accounting for 4 ± 2.6% of urinary radioactivity and 6 ± 1.1% of biliary radioactivity (Fig. 1). The metabolite resists bacterial arylsulfatase and β'-glucuronidase. Application of the HPLC technique described by Wojtowicz (10), involving a mobile phase containing NiCl2, that was useful with recoveries greater than 98% for analogues of 8-hydroxyquinoline, also failed with 5-OH-IQ. Therefore, the conjugated metabolite M-1 was isolated and rechromatographed repeatedly to give a pure sample. M-1 (about 700 µg estimated from 1C content) was hydrolyzed with β-glucuronidase in 1 ml buffer under standard conditions, the concentration of M-1 being 1.8 µM. The incubation mixture was heated briefly to 90°C to precipitate the enzyme protein, cooled to room temperature, and centrifuged. The supernatant solution was extracted five times with 2 ml each of n-butanol (HPLC grade). The butanol extracts were back-extracted with 2 ml of distilled water to remove any protein or buffer. The butanol layer, containing 59.3% of the radioactivity, was removed in a high-vacuum, maintained for 5 h to ensure a dry sample. An aliquot of the residue was used for mass spectrometry. The remainder was dissolved in DMSO-d6 for NMR spectroscopy, including determination of NOE.
z = 214 indicating the loss of 176, the molecular weight of observed at m/z = 391 (Fig. 2). The mass spectrum of the glucuronic acid conjugate.

It was labile in 1 N HCl heated at 60°C for 1 h. The hydrolysis product was identified as IQ based upon coinjection with IQ standard. The N-glucuronide conjugate of IQ, that was biosynthesized as described, coeluted with this biliary and urinary metabolite. The downfield portion of the NMR spectrum of m-1 was similar to that of IQ (Table 1). There were three unas-

Identification of M-1 as 5-OH-IQ Glucuronide. M-1 ac-

Identification of M-2 as 5-OH-IQ Sulfate Ester. M-2 was found in urine and bile in substantial amounts (26.7 to 35.4% of urinary metabolites, about 10–11% of the dose) but not in feces. This compound could be altered readily and quantitatively by heating for 15 min at pH 1, or by subjecting it to mammalian sulfatase. On the other hand, it was unchanged by mammalian or bacterial β-glucuronidase. This compound is, therefore, a sulfate ester.

The 1H-NMR spectrum (Table 1) of M-2 displays the intact resonances of the N-CH3 and NH2 group protons. The ch.

Metabolites of [5-3H]IQ. As was previously described (6), when urine collected from rats gavaged [5-3H]IQ, was analyzed by HPLC using an on-line radioflow detector, there was no significant radioactivity present at the retention time of M-1 and M-2, (Fig. 4) or even when the collected M-1 and M-2 metabolites were counted in a scintillation counter. On the other hand, 3H was present in the peaks corresponding to M-3 and M-1. Thus, biochemical substitution completely eliminated tritium from the 5-position of IQ.

DISCUSSION

The metabolism of homocyclic aromatic compounds, including important classes of carcinogens, has been clarified through

Fig. 1. The metabolites in urine of rats given [14C]IQ by gavage were resolved by HPLC on Partisil-5 ODS-3 (250 mm x 4.6 mm) using systems described in the text. In A, a small peak, m-1 (arrow) is IQ N-glucuronide, M-1 and M-2 are 5-OH-IQ glucuronide and sulfate ester, respectively. In B, the peak corresponding to m-1 is increased by coinjection of biosynthetic IQ N-glucuronide. Analysis of rat bile displays similar patterns.

Fig. 2. EI mass spectrum of M-1, 5-OH-IQ glucuronide (A) performed with ammonium chloride to assist volatilization of metabolite. (M+H)* is 391. Spectrum of M-2, 5-OH-IQ sulfate ester (M+H)* is 296 (B).

Fig. 3. CI mass spectrum of 5-OH-IQ in a methane atmosphere. (M+H)* is 215.
intensive research in the last 30 years (11-13). Novel types of heterocyclic amines were discovered in the human food chain, produced during ordinary cooking processes. Because they are among the most mutagenic chemicals known, interest in their properties, metabolism, and mode of action has led to increasing research in this field (1-3, 14, 15). These mutagens are carcinogens of the same order of potency as the human carcinogen 4-aminobiphenyl, but with a broader, yet organ-specific action, including mammary gland, intestines, pancreas, ear duct, liver, and urinary bladder (1, 13). These carcinogens are present in fried or broiled meats in amounts up to 20-180 \( \mu \text{g} / \text{kg} \) (3). Even though exposure to these genotoxic carcinogens occurs almost daily from childhood onwards in meat- or fish-eating populations, the amounts present are small, and may not be a great risk of cancer under those conditions. However, in the Western world, and increasingly in Japan, most people are also accustomed to a dietary tradition involving total fat intake of about 40% of calories, with a demonstrated potent promoting effect in specific target organs like breast, colon, and pancreas, but not liver. This phenomenon is the rationale underlying the hypothesis that the nutritionally linked neoplasms in the Western world, like breast or colon, stem both from the intake of the IQ-type carcinogens and organ-specific promoting effects associated with fat (4, 5). One mechanism of biochemical activation to reactive genotoxic compounds in rats and humans of arylamines like 4-aminobiphenyl, carcinogenic in humans, is \( N \)-oxidation, and the IQ-type carcinogens display the same activation mechanism (1, 13, 14). Systematic studies of the metabolism of two typical heterocyclic amines, IQ and MelIQx, were undertaken by several groups (6, 16-23). We now find that IQ is converted to the \( N \)-glucuronide, a minor metabolic reaction, but to a considerable extent to the 5-hydroxy derivative, excreted in bile and urine as glucuronic acid conjugate and as sulfuric acid ester (Fig. 5). Since such conjugates are hydrolyzed to the aglycone in the intestinal tract by enzymes from the bacterial flora, 5-OH-IQ should be present in feces and urine. Yet, it was noted that 5-OH-IQ could not be isolated by HPLC techniques, for the injected compound was not released as a single, clean peak. Similar difficulties were encountered with the corresponding metabolite of MelIQx studied by Turc esky et al. (20). Also, even 8-hydroxyquinoline analogues, with a similar structural relationship of the hydroxy group to the quinoline nitrogen as in 5-OH-IQ, cannot be clearly separated by HPLC (10). An eluent mixture containing \( \text{Ni}^{2+} \) was needed to permit HPLC analysis of the 8-hydroxyquinoline analogues, but our experience was that this system was ineffective for 5-OH-IQ. We, therefore, obtained the required analytical data for structural analysis on the conjugates themselves, or the pure compound derived from the purified conjugates.

The mechanism of hydroxylation of IQ most likely involves direct insertion of \( \text{OH} \) via attack by a reactive hydroxy group, through the action of specific enzymes, yet to be determined, like cytochromes P-450, mixed function oxidases, or prostaglandin synthetases. Indeed, the total loss of tritium from \( [5-\text{H}] \text{IQ} \) implies a substitution of the 5-hydrogen by hydroxyl, without an NIH shift (24). The NIH shift occurs when the initial reaction involves an epoxidation of a vicinal group like carbons 4 and 5, followed by rearrangement of the oxygen to the 4- or 5 carbons (24). This leads to migration of the hydrogen and partial retention of tritium in the heterocyclic or homocyclic molecule. Thus, the absence of tritium in the two isolated metabolites, 5-OH-IQ glucuronide and 5-OH-IQ sulfate, suggests that 5-OH-IQ is formed by direct hydroxylation of IQ (12, 25). With quinoline, benzo[\( f \]quinoline and benzo[\( h \]quinoline, on the other hand, a sequence of reactions involving an epoxide, or a dihydrodiol and then a dihydrodiol epoxide has been demonstrated (26, 27). The reactive positions appear to be in the heterocyclic part of the quinoline ring system (28). Thus, current knowledge suggests that the metabolism of IQ is mechanistically distinct from that of quinoline or the benzoquinolines.

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