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

Howard J. Luks, Thomas E. Spratt, M. Thaddeus Vavrek, Suzanne F. Roland, and John H. Weisburger

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 yielded quantitatively important metabolites.

INTRODUCTION

Heterocyclic amines typified by having a 2-amino-3-methyl or similar o-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 major polar metabolites of IQ, one minor, two major, obtained from urine, bile, and feces of rats given IQ (5, 6). Urines were collected in test tubes cooled by dry ice and methanol during the two successive 24-h periods following each dosing of IQ. Administration by Gavage. The rats (body weights about 300 g) were acclimatized in the metabolism cages for 3 days and transferred to the 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 of urine and feces and have a spill-free feeding cup. The cage was equipped with a rubber mat and a food plate. Each animal had its own drinking water bottle and a lamp for illumination from Zeigler Brothers, Gardners, PA. The cage was equipped with a rubber mat and a food plate. Each animal had its own drinking water bottle and a lamp for illumination.

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To whom requests for reprints should be addressed, at American Health Foundation, Valhalla, NY 10595.

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-hydroxylimidazo[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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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 Chemdyn 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 phosphatidylcholine (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 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 NH2 in IQ (Table 1). The mass spectrum, obtained in the presence of ammonia 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 cage 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 separate collection of urine and feces and have a spill-free feeding cup. The care of the animals was in accordance with the Guide for the Care and Use of Laboratory Animals, NIH Publication 85-23 (9).

Administration by Gavage. The rats (body weights about 300 g) 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 M HCl and adjusted to pH 3 were given by gavage. Each rat 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. The same way, [5-3H]-IQ was administered to four rats.

Urines 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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The bile duct of four rats was cannulated and exteriorized in the usual way under Nembutal anesthesia (35 mg/kg, i.p.). [2-14C]IQ (40 mg IQ/kg) was gavaged as a solution in 1 ml of 0.1 N HCl adjusted to pH of 3. The animals were placed in restrictive metabolism tubes designed to permit collection of bile, urine, and feces and maintain the fistula patent. Bile, urine and feces were obtained for successive 24-h periods.

Administration in the Diet. An aliquot of NIH-07 diet was mixed with the required amount of [2-14C]IQ (specific activity, 1.8 x 10^6 dpm/mg) and ground carefully in a mortar. The concentrate so prepared was added to a larger quantity of powdered NIH-07 diet in a 1-gallon paint can to give the desired concentration of 300 ppm. The sealed can was shaken on a paint shaker for 30 min. Effective mixing was verified by measuring the isotope concentration in three weighed samples from different levels of the can.

The amount of IQ ingested was calculated from the quantity of food consumed by each animal (including any food found in the spill-tray). After 48 h, a diet containing 300 ppm unlabeled IQ was offered for three 24-h periods, and the excreta were collected. The rats were then transferred to standard rat holding cages and given powdered diet containing 300 ppm unlabeled IQ and drinking water ad libitum for 6 weeks. After 6 weeks the rats were returned to metabolism cages and fed the 300 ppm [2-14C] IQ diet for another 48 h and unlabeled IQ for a further 72 h. Excreta were collected as in the first feeding period.

Analysis of Metabolites. Urinary and biliary metabolites were separated initially by injecting aliquots into an HPLC system using a two Waters Model 505 pump (Millipore Waters Division, Milford, MA), Model 680 automatic gradient controller, Model 810 maximal data control system configured with pump control, a Waters 440-UV detector (254 nm), and a Waters Model 712 WISP autoinjector, with two Whatman M-9 Partisil 10-ODS-3 (250 mm x 9.4 mm) columns in series. The gradient used initially for quantitative preparation consisted of linear segments, starting at 0% methanol/100% 20 mM potassium phosphate buffer (pH 6.8) for p-glucuronidase, yielding a final concentration of 750 pM of metabolite. p-glucuronidase, (1000 units), and arylsulfatase (25 units) were added to the buffered solutions of the metabolites and the mixtures were incubated at 37°C for 1.5 h. 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 of 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.

Nuclear Magnetic and Mass Spectrometry. The Instrumental Analysis Facility of this Institute (K. D. Brunemann, M. Kagan, and B. J. Misra) provided support. Proton magnetic resonance spectra in DMSO-d6 using tetramethylsilane as an internal standard were acquired with a Bruker AM360WB instrument. The NOE difference experiments were performed on about 300 gg of each compound. The N-methyl protons were irradiated for 5-20 s over approximately 3000 scans, the exact number depending on the amount of the sample. Desorption and electron impact mass spectrometry were performed on a Hewlett-Packard model 5988 mass spectrometer equipped with an HP1000 RTE operating system and RPM data processing equipment. Direct insertion mass spectrometry used a 70-eV ionization voltage, 9300-µA ionization current, a 3-kV accelerating voltage, and a source temperature of 200°C. A sample solution of 3 µg/ml of each conjugated metabolite was prepared in distilled water saturated with NH4Cl, to facilitate entrainment of the sample into the instrument chamber.

RESULTS

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-sulfamate, 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 N-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 p-glucuronidase. Therefore, the metabolite was prepared in distilled water saturated with NH4Cl, to facilitate entrainment of the sample into the instrument chamber.

MAJOR METABOLITES OF FOOD CARCINOGEN IQ

<table>
<thead>
<tr>
<th>Chemical shift downfield (ppm)</th>
<th>1H-NMR spectral parameters of IQ and metabolites</th>
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<tbody>
<tr>
<td>Assignment</td>
<td>IQ</td>
</tr>
<tr>
<td>NH</td>
<td>6.59</td>
</tr>
<tr>
<td>NH₂</td>
<td>3.75</td>
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<tr>
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<td>8.54</td>
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<tr>
<td>—COOH</td>
<td>13.06</td>
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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 provided the basis for a useful structural analysis. The base point at m/z = 214 was substituted at the C-5 position. Other than the NH2 resonance there were no D2O exchangeable proton in the spectrum, and all the other resonances characteristic of IQ were intact. An El mass spectrum yielded a molecular ion (M + H)+ of m/z = 296 (Fig. 2). The base ion at m/z = 214 was produced by the loss of a fragment of 82 amu as expected for a glucuronic acid, consistent with a hydroxy-IQ aglycone. Identification of the aglycone after enzymatic cleavage of the glucuronide was unexpectedly difficult. When the HPLC procedures for the nonpolar metabolites of IQ (6) were used, the aglycone never gave a specific peak, but rather a slow incomplete elution of radioactivity in a shallow peak extending over 12–15 min in the nonpolar region of the chromatogram.

However, isolation of the aglycone from the highly purified M-1 provided the basis for a useful structural analysis. The NMR spectrum had the characteristic resonances of IQ, except the doublets of the C-4 and C-5 protons had been replaced by a singlet. In an NOE experiment, irradiation of the N-CH3 group produced a positive 10% NOE on the singlet at 7.17 ppm. This result assigns the singlet to the C-4 proton. The remaining IQ resonances were unaffected (Table 1). The data, together with the molecular weight of 215 (Fig. 3) show that the aglycone is 5-OH-IQ, and M-1 is the glucuronic acid conjugate.

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 quantitively 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 characteristic doublets of the C-4 and C-5 protons were absent and only a singlet remained, suggesting that either the C-4 or C-5 proton had been substituted. An NOE experiment was performed by irradiation of the N-CH3 protons and a positive 20% effect was documented on the remaining singlet. This confirms that the singlet represents the C-4 proton and that the metabolite is substituted at the C-5 position. Other than the NH2 resonance there were no D2O exchangeable proton in the spectrum, and all the other resonances characteristic of IQ were intact. An El mass spectrum yielded a molecular ion (M + H)+ of m/z = 296 (Fig. 2). The base point at m/z = 214 was produced by the loss of a fragment of 82 amu as expected for a sulfate conjugate. The base ion at m/z = 214 and the results of the NMR-NOE analyses indicate that oxygen is substituted at the C-5 position.

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
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 μg/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 MeIQx, 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 MeIQx studied by Turkesky 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 Ni²⁺ 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 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 [5-3H]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 homo- cyclic 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, benzof[qu]inoline and benzo[k]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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