Metabolism of the Food-Borne Mutagen 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline in Humans

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The metabolism of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) was investigated in five human volunteers given a dietary equivalent of ^{14}C-labeled MeIQx. The amount of the dose excreted in urine ranged from 20.2% to 58.6%, with unmetabolized MeIQx accounting for 0.7−2.8% of the dose. Five principal metabolites were detected in urine, and four of the derivatives were characterized by on-line UV spectroscopy and by HPLC−MS following immunoaffinity chromatography. Two metabolites were identified as the phase II conjugates N^2-(3,8-dimethylimidazo[4,5-*f*]quinoxalin-2-yl)sulfamic acid (MeIQx-N^2-SO_3^-) and N^2-(β-1-glucosiduronyl)-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx-N^2-Gl). Two other metabolites were the cytochrome P450-mediated (P450) oxidation products 2-amino-8-(hydroxymethyl)-3-methylimidazo[4,5-*f*]quinoxaline (8-CH_2OH-MeIQx), and N^2-(β-1-glucosiduronyl)-*N*-hydroxy-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (NOH-MeIQx-N^2-Gl). The latter product is a conjugate of the genotoxic metabolite 2-(hydroxyamino)-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (NHOH-MeIQx). A large interindividual variation was observed in the metabolism and disposition of MeIQx; these four metabolites and unchanged MeIQx combined accounted for 6.3−26.7% of the total dose. The remaining principal metabolite found in all subjects accounted for 7.6−28% of the dose. It has not been previously identified in rodents or nonhuman primates, and its structure remains unknown. P450-mediated ring oxidation of MeIQx at the C-5 position, a major pathway of detoxication in rodents, was not detected in humans. Both 8-CH_2OH-MeIQx formation and NHOH-MeIQx formation are catalyzed by P450 1A2 and may be useful biomarkers of P450 1A2 activity in humans. The levels of NHOH-MeIQx-N^2-Gl found in human urine ranged from 1.4% to 10.0% of the dose, which is significantly higher than that formed in rodents and nonhuman primates undergoing cancer bioassays. Thus, bioactivation of MeIQx by P450-mediated N-oxidation is extensive in humans.

Introduction

The cooking of meat, fish, and poultry results in the formation of heterocyclic aromatic amines (HAAs)[1] (*1*−*3*). Nineteen HAAs have been identified in these cooked foods, and thus far all HAAs examined induce tumors in rodent carcinogen bioassays (*3, 4*). MeIQx is one of the most abundant HAAs found in cooked foods, and it is structurally representative of this class of chemicals (*2, 5, 6*). The diet is believed to be an important factor in the etiology of cancer (*7*), and several studies have shown that the frequent consumption of grilled meats and gravies containing HAAs is associated with an increased risk in certain types of cancers, including colorectal cancer (*8*−*12*). Thus, there is concern that HAAs may be human carcinogens.

The principal pathways of MeIQx metabolism and detoxication have been elucidated in rodents (*13*−*17*) and nonhuman primates (*18*) and include cytochrome P450

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[1] Abbreviations: HAAs, heterocyclic aromatic amines; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; NHOH-MeIQx, 2-(hydroxyamino)-3,8-dimethylimidazo[4,5-*f*]quinoxaline; NO_2-MeIQx, 2-nitro-3,8-dimethylimidazo[4,5-*f*]quinoxaline; MeIQx-N^2-SO_3^-, N^2-(3,8-dimethylimidazo[4,5-*f*]quinoxalin-2-yl)sulfamic acid; N^2-acetyl-MeIQx, 2-(acetylamino)-3,8-dimethylimidazo[4,5-*f*]quinoxaline; MeIQx-N^2-Gl, N^2-(β-1-glucosiduronyl)-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; 8-CH_2OH-MeIQx, 2-amino-8-(hydroxymethyl)-3-methylimidazo-[4,5-*f*]quinoxaline; 5-HO-MeIQx, 2-amino-5-hydroxy-3,8-dimethylimidazo-[4,5-*f*]quinoxaline; NHOH-8-(CH_2OH)-MeIQx, 2-(hydroxyamino)-8-(hydroxymethyl)-3-methylimidazo[4,5-*f*]quinoxaline; NOH-MeIQx-N^2-Gl, N^2-(β-1-glucosiduronyl)-*N*-hydroxy-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; PCB, polychlorinated biphenyls; UDPGA, uridine 5′-diphosphoglucuronic acid; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; ANF, α-naphthoflavone; CID, collision-induced dissociation.

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(P450)-mediated ring oxidation at the C-5 position, followed by conjugation to sulfate or glucuronide. Direct conjugation to the exocyclic amino group also occurs, and N^2-sulfamate and N^2-glucuronide conjugates have been detected in these species. In addition, glucuronidation of MeIQx at the N^1-imidazole moiety is a major pathway of detoxication in nonhuman primates undergoing carcinogen bioassay (*18*). Metabolic activation of MeIQx occurs by P450-mediated N-oxidation to form NHOH-MeIQx, a direct-acting mutagen that readily binds to DNA (*19*−*21*). NHOH-MeIQx is chemically unstable and has not been detected in vivo; however, the N^2-glucuronide conjugate of NHOH-MeIQx is stable and has been detected in hepatocytes, bile, and urine of rodents (*16*, *17*).

Several pathways of MeIQx metabolism have been characterized in vitro with human tissues. Human liver microsomes N-hydroxylate MeIQx and other HAAs at levels comparable to animal species which develop tumors in long-term carcinogen bioassays (*19*, *20*, *22*−*24*). Human liver and colon may further activate the *N*-hydroxy-HAAs by *N*,*O*-acetyltransferase and sulfotransferase to form highly reactive esters that bind to DNA (*23*, *25*, *26*). However, there is limited information on the metabolism and disposition of MeIQx in humans. Analysis of MeIQx in urine of humans following consumption of fried meats has shown that the absorption and metabolism of MeIQx are extensive with only 1−5% of the oral dose excreted in urine as unchanged MeIQx within 24 h (*27*−*29*). Furthermore, the amount of MeIQx recovered in the urine of subjects who were treated with the P450 1A2 inhibitor furafylline increased by as much as 14-fold (*30*), indicating that P450 1A2 is an important enzyme in metabolism of MeIQx in vivo. Another study revealed that humans transform MeIQx by phase II metabolism, and there was evidence of acid-labile MeIQx-N^2-Gl and MeIQx-N^2-SO_3^- conjugates present in urine (*29*). The MeIQx content increased by 3−21-fold following acid treatment of urine which quantitatively hydrolyzed these phase II conjugates to MeIQx, and these products combined with MeIQx accounted for as much as 14% of the ingested dose (*29*, *31*).

The direct spectroscopic characterization of MeIQx metabolites in humans has not been reported, and the major pathways of MeIQx metabolism in vivo remain to be elucidated. In this study, we have investigated the biotransformation of MeIQx in aged subjects who underwent colorectal cancer surgery. Our objective was to identify pathways of MeIQx metabolism in human urine by UV and mass spectroscopic techniques following immunoaffinity purification. The results show that several urinary metabolites of MeIQx are derived from P450 1A2-mediated oxidation and can be used as biomarkers to aid in the human risk assessment of this dietary mutagen.

Materials and Methods

Caution: *MeIQx and several of its derivatives are carcinogenic to rodents and should be handled carefully.*

Chemicals and Reagents. MeIQx and [2-^{14}C]MeIQx (10 and 47 mCi/mmol) were purchased from Toronto Research Chemicals, Inc. (Downsview, Ontario, Canada). Furafylline was obtained from Ultrafine Chemicals (Manchester, U.K.). C_{18} EC cartridges (1 g) were obtained from Machery Nagel (Düren, Germany). Human recombinant P450 1A2 microsomes (162 pmol of P450 1A2/mg of microsomal protein) derived from human B lymphoblastoid cells were purchased from Gentest Corp. (Woburn, MA). Human liver samples designated as HL102, HL103, HL112, and HL127 were provided by Dr. F. P. Guengerich, Vanderbilt University School of Medicine, Nashville, TN, and human liver sample designated as HL-G was kindly provided by Dr. F. F. Kadlubar, National Center for Toxicological Research, Jefferson, AR.

Human Study. Five human subjects who were undergoing colorectal cancer surgery volunteered for this study. A written protocol was provided to the subjects and followed-up by further discussion on the nature of the study. All subjects participated in the study after giving informed consent. The protocol of this study was independently reviewed by and approved by the Human Research Ethics Committee at York District Hospital, the Department of Health Committee on the Administration of Radioactive Substances to Persons−the Medicines (administration of radioactive substances) Regulation of 1978, United Kingdom, and the Institutional Review Board at the Lawrence Livermore National Laboratory. Each subject received [2-^{14}C]-MeIQx (4.3 μCi, 21 μg) which was placed in a gelatin capsule. This dose is equivalent to the amount of MeIQx consumed in several meals of well-done grilled beef and is comparable to the level of total HAAs consumed in the daily diet (*6*, *27*, *32*). Subjects took the capsule approximately 4−6 h prior to anesthesia and surgery (anesthetics varied with each patient and were a combination of the following: thiopentone, propofol, or etomidate; atracurium or naucuron was used as muscle relaxants). Dose adminstration was under the auspices of Dr. S. H. Leveson, York District Hospital. Urine was collected up to 26 h posttreatment and stored frozen on dry ice.

Enzyme Assays. Liver microsomes were prepared by differential centrifugation as previously described (*17*). Rates of hepatic microsomal N-oxidation were determined using microsomal protein (1 mg/mL) and [2-^{14}C]MeIQx (200 μM, 10 mCi/mmol) in 100 mM potassium phosphate buffer (pH 7.6), containing 0.5 mM EDTA, 5 mM glucose 6-phosphate, 1 unit/mL glucose 6-phosphate dehydrogenase, 0.4 mM NADPH, and 0.3 mM NAD^+. Incubation was conducted at 37 °C for 20 min. Inhibition studies were conducted with α-naphthoflavone (ANF) (10 μM) or furafylline (50 μM) where the inhibitor was preincubated with microsomes for 3 or 8 min, respectively, prior to the addition of MeIQx. The reaction was terminated by addition of 3 volumes of chilled acetonitrile followed by removal of the protein by centrifugation. The supernatant was concentrated under a stream of nitrogen, and metabolites were analyzed by HPLC with a Varian Vista 5000 system (Basel, Switzerland) equipped with a Supelco C_{18} dB column (4 μm, 4.6 × 250 mm) (Buchs, Switzerland) connected to a Berthold LB 506 C-1 radioactivity monitor (Regensdorf, Switzerland). Metabolites were eluted at a flow rate of 1 mL/min using a solvent of 50 mM $NH_4CH_3CO_2$ buffer (pH 6.8) and 20% (v/v) CH_3OH for 15 min, followed by a linear gradient to 100% CH_3OH at 25 min, and held at 100% CH_3OH for 5 min.

Urinary Metabolite Purification and Analysis. Aliquots of urine (10 mL) were added to 5 volumes of methanol−acetone (1:1, v/v) on ice for 1 h. Protein and salt were removed by centrifugation at 10000*g* for 20 min at 4 °C. The supernatant was rotary evaporated to dryness and resuspended in 50 mM $NH_4CH_3CO_2$ buffer (pH 6.8) This extract was further purified by solid-phase extraction with a C_{18} cartridge (*29*). The recovery of radioactivity was 84 ± 14% (mean ± SD, n = 10). After evaporation to dryness, the extract was analyzed by HPLC with a Varian Vista 5000 system equipped with a Supelco C_{18} dB column (4 μm, 4.6 × 250 mm). Metabolites were eluted at a flow rate of 1 mL/min using a solvent of 50 mM $NH_4CH_3CO_2$ buffer (pH 5.0) and 11% (v/v) CH_3OH for 10 min. This was followed by a linear gradient which reached 20% (v/v) CH_3OH at 40 min, followed by an increase to 100% CH_3OH at 51 min, and held at 100% CH_3OH for an additional 5 min. Fractions were collected (30 s) and measured by liquid scintillation counting.

Table 1. Patient Diet, Drug, and Smoking Histories

subject	age (years)	sex	drugs	smoking history	diet
1	63	M	no medication	exsmoker, does not come into contact with other smokers	medium-cooked meat, either fried or roasted, >5 times/week
2	84	M	ferrous sulfate, 200 mg, 1/day	never smoked, does not come into contact with other smokers	medium-done roasted meat in casseroles, 3−5 times/week
3	70	F	no medication	smoker, does not come into contact with other smokers	well-done roasted meat in casseroles, 3−5 times/week
4	80	M	becotide v, intal, salbutamol	exsmoker, regularly comes into contact with other smokers	well-done meat either grilled or fried, every day
5	58	M	aspirin, 1/day	exsmoker, regularly comes into contact with other smokers	well-done grilled meat, 3−5 times/week

Urinary metabolites partially purified by solid-phase extraction were further purified by immunoaffinity chromatography as previously reported (*33*). The immunopurified metabolites were analyzed by HPLC as described above except that a Hewlett-Packard 1090M HPLC (Geneva, Switzerland) equipped with a diode array UV/vis detection system was employed for on-line UV spectra acquistion. Metabolites were further characterized by treatment with β-glucuronidase or arylsulfatase or by acid hydrolysis in 1 or 6 N HCl as previously described (*14*, *16*).

Spectroscopic Analyses. HPLC−MS analyses were carried out on Finnigan MAT TSQ-700 and TSQ-7000 mass spectrometers (Bremen, Germany) connected via a Finnigan electrospray interface to a Waters 600-MS pump (Rupperswil, Switzerland) or a Hewlett-Packard 1100 pump, respectively. Separation of microsomal metabolites was done with the same HPLC conditions as described above in Enzyme Assays. The solvent was split 1:10 with a LC Packings Acurate microflow processor (Omnilab, Commugny, Switzerland) before the mass spectrometer and 100 μL of solvent entered the source. The electrospray interface was operated with a high voltage of 4.5 kV and a capillary temperature of 200 °C. Nitrogen was used as sheath gas at a pressure of 80 psi. The mass spectrometer operated in positive detection mode, using full scan mode from 50 to 400 Da. Analysis of human urinary metabolites following immunoaffinity purification was performed as described above for radiolabeled analyses except that the concentration of NH_4-CH_3CO_2 buffer was decreased to 10 mM. The effluent was also split 1:10 before entering the MS, and a solution of acetic acid (10%, v/v) was added via a two-way mixing tee post HPLC column at a flow rate of 2 μL/min. MS/MS analyses of the metabolites were carried out on the Finnigan TSQ-7000 mass spectrometer after collision-induced dissociation (CID) of the protonated molecular ions of each of the metabolites. A collision energy of 20 eV was used for metabolites and 30 eV for MeIQx. Argon was used as the collision gas at a pressure of 2.5 mTorr. The capillary temperature was set at 220 °C. The capillary voltage and nitrogen sheath gas were operated under the conditions described above.

Electron impact mass spectra were recorded from 20 to 500 Da at 70 eV on a Finnigan MAT 8430 double focusing mass spectrometer working with an EI source heated at 180 °C.

^{1}H NMR characterization of 8-CH_2OH-MeIQx was performed at 360 MHz with a Bruker AM-360 spectrometer (Spectrospin AG, Fällanden, Switzerland) equipped with a 5-mm ^{1}H probehead under conditions previously described (*16*). The NMR sample was prepared in DMSO-d_6 with TMS as an internal standard.

Reference Standard Metabolites. NHOH-MeIQx was prepared by reduction of NO_2-MeIQx with ascorbic acid and purified as previously described (*23*). MeIQx-N^2-SO_3^- was prepared by treatment of MeIQx with chlorosulfonic acid (*14*). N^2-Acetyl-MeIQx was prepared by reaction of MeIQx with acetic anhydride as previously described (*23*). NOH-MeIQx-N^2-Gl and MeIQx-N^2-Gl were prepared by incubating 2 mg of NHOH-MeIQx with 30 mg of liver S-9 protein from rats pretreated with PCB (*17*) in 5 mL of 100 mM KH_2PO_4, 0.5 mM EDTA (pH 7.6) containing 5 mM $MgCl_2$ and 20 mM UDPGA for 3 h at 37 °C. 8-CH_2OH-MeIQx was prepared by incubating 1 mg of MeIQx with 30 mg of liver S-9 protein from rats pretreated with PCB in 4 mL of 100 mM KH_2PO_4 buffer (pH 7.6) containing 5 mM glucose 6-phosphate, 1 mM NADPH, 1 mM NAD^+, and 10 U of glucose 6-phosphate dehydrogenase for 3 h at 37 °C. The 5-*O*-glucuronide and 5-sulfate conjugates of 5-HO-MeIQx were prepared by incubating MeIQx with rat liver S-9 protein as above except that 10 mM PAPS or 20 mM UDPGA was added as cofactors. The metabolites were purified and characterized under conditions previously described (*16*). Comparable experiments were conducted with MeIQx and human liver S-9 protein which showed that the 5-*O*-glucuronide and 5-sulfate conjugates of 5-HO-MeIQx were not formed.

Table 2. Percent Dose Recovered in Urine as a Function of Time[a]

subject	time 1 (h)	% dose	time 2 (h)	% cumulative dose	time 3 (h)	% cumulative dose
1	6	12.6	12	19.6	24	20.2
2	4	12.6	6	36.8	26	46.0
3	4	36.4	12	37.6	25	41.4
4	5.5	47.6	12	58.6	24	58.6
5	3.5	26.0	12	35.6	25	38.6

[a] Time after the administration of MeIQx. The first time point was obtained prior to surgery.

^{1}H NMR was conducted on approximately 40 μg of 8-CH_2OH-MeIQx obtained from the rat S-9 incubation. The ^{1}H NMR spectrum supported this proposed structure; the C-8 methyl group of MeIQx at 2.74 ppm was absent, and a new signal which integrated to two protons was found at 4.80 ppm. It occurred as a singlet due to fast hydroxyl hydrogen exchange in solution. All other protons of the MeIQx moiety (*16*) were detected [3.66 ppm, s, 3H, NCH_3; 6.56 ppm, s, ca. 2H, D_2O exchangeable, NH_2; 7.59 ppm, d, 1H, J = 8.6 Hz, H-5 (or H-4); 7.77 ppm, d, 1H, J = 8.7 Hz, H-4 (or H-5); 8.85 ppm, s, 1H, H-7]. The small amount of sample did not permit NOE experiments to make the assignments of the H-5 and H-4 protons unequivocal. The electron impact mass spectrum also supported the structure with a molecular ion detected at m/z 229.

Results

The diet, drug, and smoking histories of the subjects are summarized in Table 1. All of the subjects consumed cooked meat at least 3−5 times per week. Several subjects were on medication, and four of the five volunteers have a smoking record. The absorption of [^{14}C]-MeIQx was rapid in all five subjects, and between 20.2% and 58.6% of the administered dose was eliminated in urine within 24 h, with 90% of the recovered dose excreted within 12 h of treatment (Table 2). The rapid absorption and elimination of MeIQx are in agreement with previous reports (*27*−*29*).

Five principal metabolites were found in urine of all subjects (Figure 1). Four of these metabolites were previously identified in rodents and nonhuman primates (*13*−*18*), and their structures are shown in Chart 1. The

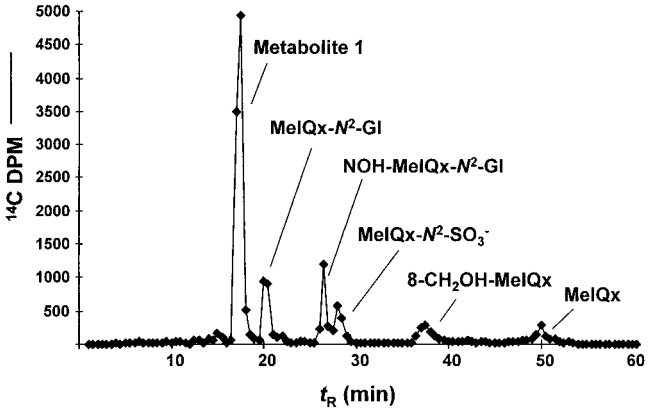
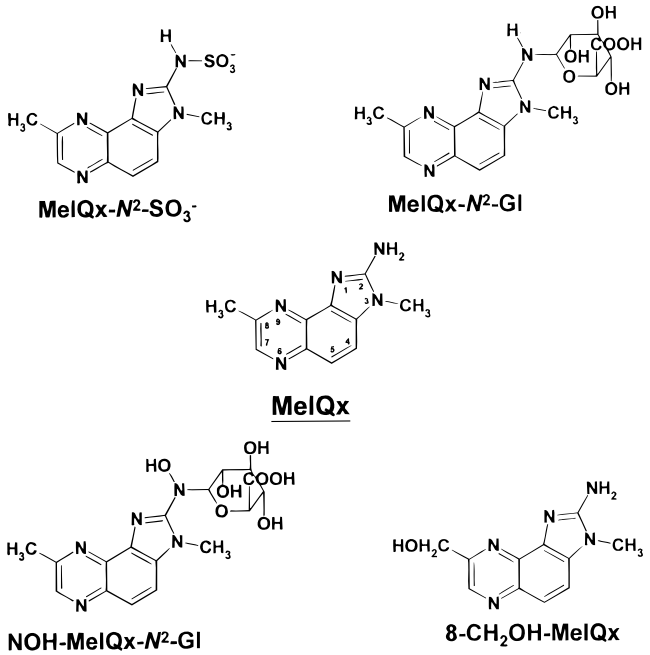


Figure 1. HPLC radiochromatogram of metabolites of MeIQx in urine of a subject collected over 26 h. Under these chromatographic conditions, the retention times of the 5-*O*-glucuronide and 5-sulfate conjugates of 5-HO-MeIQx and N^2-acetyl-MeIQx were, respectively, 14, 23, and 47 min.

Chart 1. Chemical Structures of MeIQx and Known Metabolites Found in Human Urine



major metabolite (metabolite 1) found in urine of all subjects has not been detected in other species. The 5-*O*-glucuronide and 5-sulfate conjugates of 5-HO-MeIQx, which are prominent metabolites in urine and bile of rats and nonhuman primates (*15*, *17*, *18*), were not detected in human urine. In addition, N^2-acetyl-MeIQx, which has been detected as a minor metabolite in urine of rats (*13*), was not excreted in urine of these human subjects.

There was a large interindividual variation in the quantities of urinary excretion of MeIQx and its metabolites (Table 3). Unchanged MeIQx accounted for 0.7−2.8% of the dose. Metabolite 1, the prominent metabolite in all subjects, accounted for 7.6−28.0% of the dose. The ranges of MeIQx-N^2-Gl and MeIQx-N^2-SO_3^- excreted in urine were 1.6−6.3% and 0.6−3.1% of the dose, respectively. The amount of the NOH-MeIQx-N^2-Gl excreted in urine accounted for 1.4−10.0% of the dose. The levels of this metabolite were highest in subjects 2, 4, and 5 who also had the highest amounts of 8-CH_2OH-MeIQx (1.0−4.4% of the dose).

MeIQx and its metabolites were characterized by UV and mass spectroscopy following immunoaffinity purification in order to unambiguously confirm the proposed structures. MeIQx and four of the metabolites were recognized by the monoclonal antibodies, and purified by immunoaffinity chromatography. Metabolite 1 was not recognized by the monoclonal antibodies, and it was resistant to enzymatic hydrolysis with β-glucuronidase and arylsulfatase (*14*, *16*, *17*). Acid treatment (1 or 6 N HCl at 100 °C for 18 h) did not modify the retention time of this derivative by HPLC, whereas MeIQx-N^2-Gl and MeIQx-N^2-SO_3^- were hydrolyzed under the same conditions with quantitative regeneration of MeIQx.

The identities of the immunopurified metabolites were substantiated by HPLC comigration with standards prepared chemically or biosynthetically from rodent tissues. The on-line UV spectra of the human metabolites are superimposed with their rodent counterparts and are in excellent agreement (Figure 2). The spectra of MeIQx-N^2-SO_3^- and 8-CH_2OH-MeIQx are readily distinguished from each other and from the glucuronide metabolites, with respect to their maxima and minima. However, the UV spectra of MeIQx-N^2-Gl and NOH-MeIQx-N^2-Gl are nearly identical except that the absorbance maximum of NOH-MeIQx-N^2-Gl has been shifted by 2 nm from 274 to 272 nm.

The metabolites' identities were further corroborated by HPLC−MS/MS using selective reaction monitoring. Table 4 summarizes the characteristic ions in the MS/MS spectra of the reference standard MeIQx metabolites obtained after CID of their protonated molecular ions. The MS/MS spectrum of 8-CH_2OH-MeIQx is characterized by an intense fragment at *m/z* 212 corresponding to the loss of water from the protonated molecular ion. In contrast, the CID of NOH-MeIQx-N^2-Gl leads to the loss of OH from the protonated molecular ion at *m/z* 406 as well as loss of the glucuronide ($[M + H - 176]^+$) to produce protonated NHOH-MeIQx at *m/z* 230 as the base peak. For MeIQx-N^2-Gl and MeIQx-N^2-SO_3^-, the CID process generated the protonated parent MeIQx molecule at *m/z* 214 as the base peak of both MS/MS spectra. These specific fragmentations were used to scan the metabolites purified from human urine, taking into account the 2 amu mass increase due to the ^{14}C-label. Figure 3 shows the electrospray HPLC−MS/MS detection of the MeIQx metabolites in human urine following immunoaffinity purification.

Metabolism studies were undertaken in vitro using human liver microsomes and microsomes of recombinant human P450 1A2 (lymphoblastoid cells) to determine whether the unknown urinary metabolite 1 is derived from P450-mediated metabolism and to determine if P450 1A2, the principal P450 involved in N-oxidation of MeIQx and other HAAs (*22*−*24*, *34*), is also involved in the hydroxylation of the 8-CH_3 position of MeIQx. Three metabolites were formed by human liver microsomes and microsomes containing recombinant human P450 1A2. The HPLC profile and full scan mass spectra of the metabolites derived from human liver microsomes are shown in Figure 4. NHOH-MeIQx was the major metabolite formed in both microsomal preparations which is in agreement with previous reports (*23*, *24*). The minor metabolite was identified as 8-CH_2OH-MeIQx, while the structure of the remaining metabolite was tentatively assigned as NHOH-8-(CH_2OH)-MeIQx. The chemical assignments of NHOH-MeIQx and 8-CH_2OH-MeIQx were

Table 3. Distribution of MeIQx Metabolites in Human Urine: Percent of Dose Excreted in Urine over 26 h[a]

subject	unknown 1	MeIQx-N^2-Gl	NOH-MeIQx-N^2-Gl	MeIQx-N^2-SO_3^-	8-CH_2OH-MeIQx	MeIQx	% dose in urine
1	7.6	1.6	1.9	0.6	1.0	1.2	20.2
2	23.5	5.8	3.5	1.9	2.2	1.2	46.0
3	28.0	3.7	1.4	0.8	1.3	0.7	41.4
4	18.6	6.3	10.0	3.1	4.4	2.8	58.6
5	17.6	4.5	4.1	2.2	2.5	1.7	38.6

[a] The recovery of radioactivity from urine of five subjects (five urine samples in duplicate) determined following protein precipitation and solid-phase extraction was 84 ± 14% (mean ± SD, $n = 10$). The metabolite estimates were the average from two independent measurements per subject and are normalized to 100% recovery. The duplicate values were within 10%. Spiking experiments of radiolabeled metabolites at comparable levels in urine revealed that all metabolites were recovered at levels ≥85%.

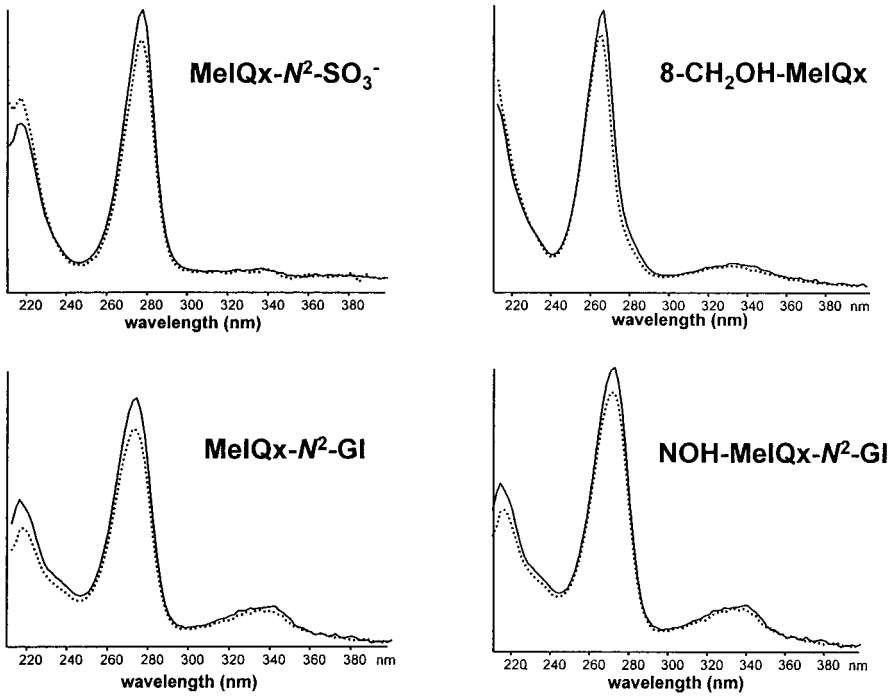


Figure 2. On-line HPLC UV spectra of four known human urinary metabolites (dashed lines) superimposed with rodent metabolites (solid lines). The HPLC buffer contained 50 mM NH_4CO_2 (pH 5.0) and CH_3OH.

Table 4. MS/MS Spectra of the Reference Standard Metabolites

metabolite[a,b]	$[M + H]^+$	$[M + H - H_2O]^+$	$[M + H - OH]^+$	$[M + H - Gl]^+$	$[M + H - SO_3]^+$	other daughter ions
MeIQx-N^2-Gl	390 (61)	372 (5)		214 (100)		286 (5), 256 (18)
NOH-MeIQx-N^2-Gl	406 (40)		389 (20)	230 (100)		242 (70), 213 (30)
8-CH_2OH-MeIQx	230 (20)	212 (100)				197 (9)
MeIQx-N^2-SO_3H	294 (63)				214 (100)	

[a] Spectra were obtained after CID at 20 eV of the protonated molecular ions. Values as *m/z* (relative intensity). [b] The spectrum of MeIQx was obtained at 30 eV with $[M + H]^+$ at 45% relative intensity and $[M + H - CH_3]^+$ $m/z = 199$ detected as the base peak.

based upon comigration and by comparison of the mass spectra and UV spectra with synthetic standards (Figure 4). The assignment of NHOH-8-(CH_2OH)-MeIQx was based upon the mass spectrum which displayed a protonated molecular ion at *m/z* 246 associated with adduct ions at *m/z* 268 ($[M + Na]^+$) and 284 ($[M + K]^+$). This indicates incorporation of two oxygen atoms in the MeIQx molecule. The mass spectrum showed a loss of oxygen ($[M + H - OH]^+$) at *m/z* 229 which is characteristic of FAB and electrospray mass spectra of NHOH-MeIQx and not characteristic of the spectra of metabolites hydroxylated directly to the heterocyclic ring of MeIQx (*16*, *17*, *35*). The base peak was observed at *m/z* 214 ($[M + H - 32]^+$) which is suggestive of a loss of CH_3OH; however, this loss of M − 32 was not seen in the spectrum of 8-CH_2OH-MeIQx under these solvent conditions [50 mM $NH_4CH_3CO_2$ buffer (pH 6.8), 20% CH_3OH]. Further support for this structure was based upon the UV spectra. Hydroxylation of MeIQx at the 8-methyl position shifted the absorbance maximum of MeIQx by 2 nm from 276 to 278 nm. The absorbance maximum of the proposed NHOH-8-(CH_2OH)-MeIQx also increased by 2 nm relative to NHOH-MeIQx from 274 to 276 nm. The low-intensity maximum of this new metabolite at 345 nm and the spectral changes observed at the minimum centered at 300−310 nm are characteristic of NHOH-MeIQx. In contrast, UV spectra of metabolites hydroxylated directly to the heterocyclic ring system of MeIQx result in other spectral perturbations (*14*, *16*). The proposed NHOH-8-(CH_2OH)-MeIQx metabolite was present at higher levels than 8-CH_2OH-MeIQx in the microsomal incubations, irrespective of the incubation time (5−180 min) or substrate concentration (10−200 μM), suggesting that it is derived from NHOH-MeIQx. Metabolite 1 in human

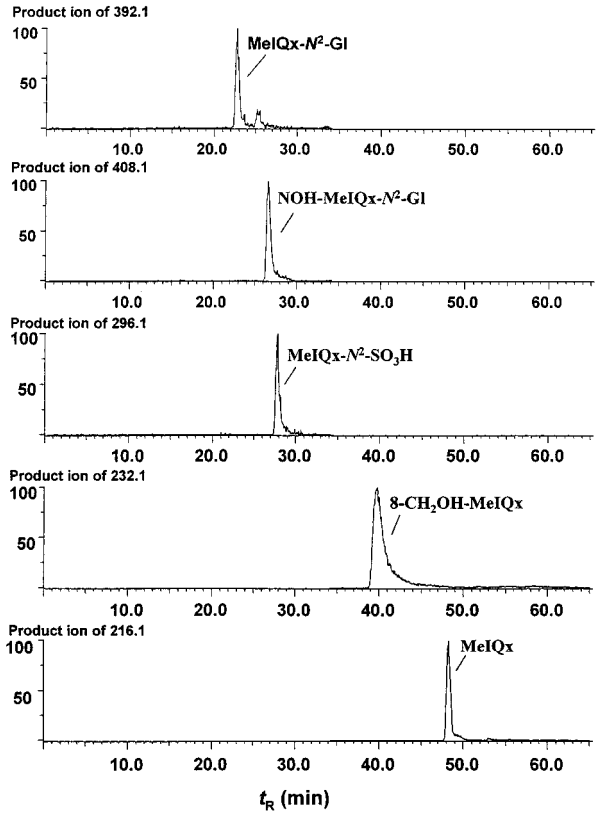


Figure 3. Selected reaction monitoring electrospray HPLC−MS/MS profile of MeIQx metabolites immuopurified from human urine. The daughter ions of MeIQ-N^2-Gl and NOH-MeIQx-N^2-Gl were $[M + H - Gl]^+$, MeIQx-N^2-SO_3H $[M + H - SO_3]^+$, 8-CH_2OH-MeIQx $[M + H - H_2O]^+$, and MeIQx $[M + H - CH_3]^+$. The $[M + H]^+$ and daughter ions were selected at the *m/z* for the corresponding ^{14}C-labeled isotope metabolites, or 2 Da higher than the mass of the unlabeled metabolites (see Table 3).

urine elutes at t_R 3.5−4 min under the chromatographic conditions employed for the in vitro microsomal metabolite analysis. Therefore, the proposed NHOH-8-(CH_2OH)-MeIQx metabolite cannot be metabolite 1. Furthermore, NHOH-(8-CH_2OH)-MeIQx and NHOH-MeIQx are chemically unstable and oxidize overtime, while urinary metabolite 1 is chemically stable.

The rates of N-oxidation and 8-hydroxylation of MeIQx by five different human liver microsome preparations varied by approximately 4-fold (Table 5). The presence of ANF (10 μM) or furafylline (50 μM), reported selective inhibitors of P450 1A2 (*30, 36*), decreased the formation of NHOH-MeIQx, 8-CH_2OH-MeIQx, and NHOH-8-(CH_2OH)-MeIQx by greater than 95% in human liver microsomes and in recombinant human P450 1A2 preparations. Thus, P450 1A2 is involved in the hydroxylation of the 8-methyl group and N-oxidation of the exocyclic amino group of MeIQx.

Discussion

Humans are exposed to HAAs daily through the diet (*1*−*4*), and epidemiology studies indicate that frequent consumption of grilled meats and gravies containing HAAs increases the risk in certain types of cancer (*8*−*12*). It is difficult to assess the contribution of HAAs to the etiology of human cancers, and biomarkers are required to assess metabolism, the biologically effective dose, and the impact of polymorphisms on HAA genotoxicity in vivo. Our data provide the first direct spectroscopic characterization of MeIQx metabolites in human urine which can be used as biomarkers for interspecies comparisons and risk assessment.

The extrapolation of the results from this study to data obtained from rodents, nonhuman primates, and healthy

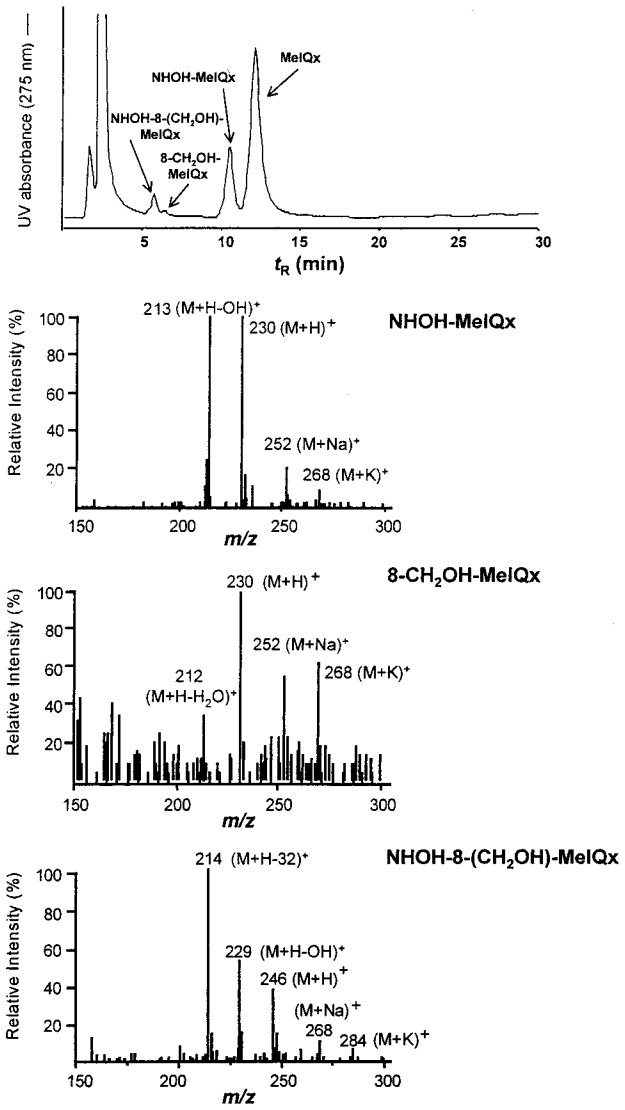


Figure 4. HPLC−UV profile of MeIQx metabolites generated in vitro from human liver microsomes after a 30-min incubation at 37 °C and electrospray full scan mass spectra of microsomal metabolites NHOH-MeIQx, 8-CH_2OH-MeIQx, and NHOH-8-(CH_2OH)-MeIQx* whose proposed structure is based upon mass and UV spectral data.

Table 5. Microsomal Metabolism of MeIQx with Human Liver Microsomes and Microsomes of Human B Lymphoblastoid Cells Containing Recombinant P450 1A2

	nmol of metabolite formed/min/mg of microsomal protein		
sample code	NHOH-(8-CH_2OH)-MeIQx	8-CH_2OH-MeIQx	NHOH-MeIQx
HL102	0.15 ± 0.04	0.04 ± 0.01	0.76 ± 0.08
HL103	0.11 ± 0.07	0.02 ± 0.01	0.65 ± 0.05
HL112	0.16 ± 0.01	0.05 ± 0.01	1.34 ± 0.10
HL127	0.15 ± 0.01	0.04 ± 0.01	0.95 ± 0.03
HLG	0.37 ± 0.04	0.07 ± 0.01	2.23 ± 0.12
recombinant[a] P450 1A2	0.12 ± 0.03	0.03 ± 0.01	0.98 ± 0.11

[a] Recombinant P450 1A2 microsomes contained 162 pmol of P450 1A2/mg of microsomal protein.

human subjects must be made with caution. In this study, the volunteers underwent colorectal cancer surgery and several of the subjects were elderly. MeIQx was given in capsule form and not as part of the diet. In addition, some of the subjects were under medication prior to surgery. All of these factors, combined with the anesthetics and surgery 4−6 h following treatment with MeIQx, could have influenced the metabolism and disposition of MeIQx. Despite these caveats, several features on the biodisposition of MeIQx in these subjects are similar to those reported on healthy human subjects (*27*−*29*) and other species (*15, 17, 18*). In this study, the amount of unchanged MeIQx recovered in urine 24 h following an oral dose of MeIQx ranged from 0.7% to 2.8%, which is comparable to the percent of unmetabolized MeIQx excreted in urine of subjects who consumed this HAA as part of a meat or fish diet (*27*−*29*). The urinary MeIQx-N^2-Gl and MeIQx-N^2-SO_3^- accounted for, respectively, 4.4 ± 1.9% and 1.7 ± 1.0% (mean ± SD, n = 5 subjects) of the dose which is comparable to the levels found in urine of healthy subjects measured indirectly as acid-labile conjugates of MeIQx following consumption of grilled meats (*29, 31*). The amounts of unchanged MeIQx, MeIQx-N^2-Gl, and MeIQx-N^2-SO_3^- excreted in urine of male rats given an oral dose of MeIQx (10 μg/kg) (*17*) were also similar. Thus, the relative contribution of these phase II pathways in detoxication of MeIQx is similar in rats and humans.

There are several important differences in the metabolism of MeIQx by humans, rodents, and nonhuman primates. The prominent metabolite of MeIQx found in the urine of human subjects, which accounts for as much as 28% of the dose, has not been detected in other species. Pharmacokinetic studies with the selective P450 1A2 inhibitor furafylline revealed that P450 1A2-catalyzed metabolism of MeIQx in humans accounts for as much as 91% of the elimination of ingested MeIQx (*30*), suggesting that this novel metabolite 1 may be a P450 1A2-derived species. However, this product is not readily formed in vitro by oxidation of MeIQx with human microsomes (Figure 4, Table 5) or human liver homogenates fortified with various cofactors. Mercapturic acid conjugates derived from reaction with the carcinogenic metabolites NHOH-MeIQx or *N*-acetoxy-MeIQx were prepared; however, the reaction products with *N*-acetylcysteine (or cysteine) do not have a similar HPLC retention time as metabolite 1 (R. Turesky, unpublished data).

Metabolite 1 was not recognized by the monoclonal antibodies, and it could not be purified from urine by immunoaffinity chromatography. We isolated metabolite 1 from 1 L of human urine using five different HPLC purification steps and attempted characterization by electron impact and FAB/MS, techniques which have been successfully used for the characterization of other MeIQx metabolites (*35*). However, meaningful spectra were not obtained. Using LC/MS, a full scan spectrum was acquired in positive ionization mode (HPLC solvent A, 2% CH_3CN in 0.1% CF_3COOH; solvent B, 0.1% CF_3COOH in 95% CH_3CN; gradient from 10% to 90% B in 20 min), which displayed a protonated molecular ion at m/z 244 and an associated adduct at m/z 285 ($[M + CH_3CN + H]^+$). In addition, ^{14}C-isotopic peaks were detected, respectively, at m/z 246 and 287. These data are suggestive of a dioxygenated metabolite, and different structures could be postulated. It will be necessary to purify this metabolite from larger volumes of urine to fully characterize it by ^1H NMR and mass spectrosopic methods.

Another important metabolic feature which shows strong interspecies differences is P450-mediated ring oxidation of MeIQx at the C-5 position. This pathway of MeIQx detoxication occurs in rodents and nonhuman primates (*15*−*18*), but it does not appear to occur in humans. In rats, the 5-sulfate and 5-*O*-glucuronide conjugates of 5-hydroxy-MeIQx combined accounted for approximately 50% of the MeIQx dose excreted in bile and urine (*17*). We observed that both conjugates were readily formed in vitro by incubation of MeIQx with rat liver S-9 preparations fortified with PAPS and UDPGA. In contrast, 5-HO-MeIQx was not formed by oxidation of MeIQx with human liver microsomes (*23, 24*) or recombinant human P450 1A2 (Table 5), and neither the 5-*O*-glucuronide nor 5-sulfate conjugates of 5-HO-MeIQx was detected following incubation of MeIQx with human liver S-9 preparations fortified with PAPS and UDPGA. In agreement with these data, neither conjugate was detected in the five human urine samples examined (Figure 1, Table 2). The absence of this oxidation pathway in human liver suggests that P450 1A1, which is active in aromatic ring hydroxylation (*37*) but not expressed in human liver (*36*−*38*), may be involved. Alternatively, the regioselectivity of human and rat P450 1A2 may be different, and rat P450 1A2 may catalyze both N-oxidation and 5-hydroxylation of MeIQx. This question is currently under investigation.

N-Oxidation of MeIQx and other HAAs by P450 1A2 is the metabolic pathway responsible for DNA adduct formation and mutagenesis (*19*−*21, 34*). The C-8 deoxyguanosine DNA adduct of MeIQx has been detected in animals (*39*) and recently identified in human kidney and colon biopsy samples by ^{32}P-postlabeling (*40*). This adduct is currently the only biomarker which can directly measure the genetic damage of MeIQx. However, this procedure requires a biopsy sample and cannot be performed routinely on a large number of healthy subjects. Therefore, the measurement of NOH-MeIQx-N^2-Gl which can be obtained noninvasively and can assess the capacity of individuals to metabolically activate this procarcinogen is of critical importance.

Glucuronidation of *N*-hydroxyarylamines is a means of transport of the carcinogenic *N*-hydroxy metabolites to tissues such as the colon and urinary bladder where hydrolysis may occur, and the liberated *N*-hydroxy derivatives can then react with DNA (*41*). In contrast to acid-abile N^2-glucuronides of *N*-hydroxyarylamines, NOH-MeIQx-N^2-Gl is stable and not hydrolyzed under the mildly acidic pH of urine (*16*). The range of NOH-MeIQx-N^2-Gl recovered in urine of humans was 1.4−10.0% of the total dose (Table 2), which is 5−10-fold greater than the amount excreted in urine of rodents (*17*) and nonhuman primates undergoing carcinogen bioassay with MeIQx (*18*) or the structurally related analogue IQ (*42*). Thus, P450 1A2-mediated N-oxidation of MeIQx in humans appears to be considerably higher than in other species; however, interspecies differences in glucuronidation rates of NHOH-MeIQx (*43*) or in the proportion of NOH-MeIQx-N^2-Gl excreted in urine must also be considered.

Metabolic polymorphisms are believed to be an important determinant of carcinogen susceptibility (*44*−*47*). There is considerable human variability in the expression

and enzyme activity of P450 1A2 which is known to activate a variety of potential human carcinogens including HAAs (*34*, *38*, *45*). Interindividual differences in human hepatic microsomal P450 1A2-mediated N-oxidation rates of the aromatic amines 4-aminobiphenyl and 2-naphthylamine have been reported where rates varied by 44-fold (*48*). Similar results were reported on the metabolic activation of several HAAs by human liver (*22*). A large variability of P450 1A2 activity also has been demonstrated in vivo where P450 1A2-mediated caffeine 3-demethylation and phenacetin *O*-deethylation varied by 50−70-fold in different subjects (*49*−*51*). In this pilot study with five human subjects, the urinary NOH-MeIQx-N^2-Gl levels differed by 7-fold (Table 3). This is similar to the extent of variation of MeIQx N-oxidation rates observed in vitro with five different human hepatic microsome preparations (Table 5), which may be reflective of variable P450 1A2 levels.

Because the bioactivation of MeIQx appears higher in these human subjects than in animal species which succumb to tumorigenesis in long-term feeding studies, the human health risk of MeIQx and other HAAs may be underestimated (*52*, *53*). Metabolism and biomonitoring studies with healthy individuals who consume MeIQx and other HAAs as part of the daily diet are warranted as part of the risk assessment process. The urinary NHOH-MeIQx-N^2-Gl metabolite provides a measure of the individual's capacity to metabolically activate this HAA and thus a measure of P450 1A2 activity. The measurement of urinary NOH-MeIQx-N^2-Gl and DNA adduct formation as indices of P450 1A2 N-oxidation and genetic damage, combined with phenotypic and genotypic markers of enzyme polymorphisms, may aid in further assessing the human health risk of these dietary mutagens.

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