

N-Oxidative Metabolism of 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) in Humans: Excretion of the *N*²-Glucuronide Conjugate of 2-Hydroxyamino-MeIQx in Urine¹

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ABSTRACT

2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), a major heterocyclic aromatic amine (HAA) formed in cooked meats, is metabolically transformed to mutagenic/carcinogenic intermediates. Cytochrome P4501A2 (CYP1A2)-mediated *N*-hydroxylation followed by phase II *O*-esterification by *N*-acetyltransferase (NAT2) are generally regarded as activation processes in which MeIQx and other HAAs are converted to genotoxic species. In this study, we determined the relationship between the activities of these two enzymes and the urinary excretion level of the *N*²-glucuronide conjugate of 2-hydroxyamino-MeIQx—*N*²-(β -1-glucosiduronyl)-2-hydroxyamino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (*N*-OH-MeIQx-*N*²-glucuronide)—among healthy subjects fed a uniform diet containing high-temperature cooked meat. The individuals ($n = 66$) in the study ate meat containing known amounts of MeIQx, and urine was collected from 0 to 12 h after the meal. After addition of the deuterium-labeled internal standard to urine, *N*-OH-MeIQx-*N*²-glucuronide was isolated using solid-phase extraction and immunoaffinity separation. The isolated conjugate was converted to the deaminated product 2-hydroxy-3,8-dimethylimidazo[4,5-*f*]quinoxaline (2-OH-MeIQx) by heating with acetic acid. 2-OH-MeIQx and its deuterated analogue were derivatized to form the corresponding 3,5-*bis*(trifluoromethyl)benzyl ether derivatives and analyzed by capillary gas chromatography-negative ion chemical ionization mass spectrometry using selected ion monitoring procedures. The subjects in the study excreted an average of $9.4 \pm 3.0\%$ (\pm SD) of an ingested dose of MeIQx as *N*-OH-MeIQx-*N*²-glucuronide in urine; the range varied from 2.2 to 17.1%. A significant correlation was found between the level of *N*-OH-MeIQx-*N*²-glucuronide in urine and the amount of MeIQx ingested ($r_s = 0.44$; $P = 0.0002$). The excretion level of *N*-OH-MeIQx-*N*²-glucuronide in urine was not associated with the enzyme activities of NAT2 or CYP1A2. This is expected with the latter enzyme because the metabolism of MeIQx is first order and very rapid at the amounts ingested. The amount of *N*-OH-MeIQx-*N*²-glucuronide in urine was not correlated with the age or sex of the individuals. Our results indicate that biotransformation of MeIQx via CYP1A2 oxidation to form the *N*-hydroxylamine followed by *N*²-glucuronidation is a general pathway of MeIQx metabolism in humans; the variability in the excreted levels of *N*-OH-MeIQx-*N*²-glucuronide is probably due to interindividual differences in UDP-glucuronosyltransferase activity and/or excretion pathways.

INTRODUCTION

The HAAs³ comprise a class of mutagenic and carcinogenic compounds produced in the low parts-per-billion range in high-temperature

cooked meats (1, 2). Humans are routinely exposed to food-derived HAAs, and there is a concern that these compounds may be dietary carcinogens (3, 4). HAAs are thought to be bioactivated to mutagenic/carcinogenic intermediates by an initial *N*-oxidation in the liver mediated primarily by CYP1A2 (5–7). Further activation of the *N*-hydroxylamine metabolite generally occurs by way of enzymatic esterification reactions catalyzed by acetyltransferases (NAT enzymes) or sulfotransferases (8–12). These more reactive metabolites can covalently bind to DNA (5, 8, 10). Alternatively, the *N*-hydroxylamine metabolites of the HAAs may undergo phase II conjugation reactions via UDP-glucuronosyltransferase(s) to form the corresponding *N*-glucuronide conjugates (13, 14). This is considered to be a significant detoxification pathway for *N*-hydroxy HAAs (14) and was found in *in vitro* studies to limit the extent of HAA-induced DNA damage (15). In addition to this enzymic pathway, glutathione *S*-transferases and glutathione can inhibit the covalent reaction of the activated derivative of HAAs with DNA (16). Thus, HAAs are capable of undergoing a complex interaction of metabolic pathways in which detoxification reactions compete with bioactivation processes (14, 17, 18). As a consequence, the balance between these two processes may play a central role in the potency of a HAA and thereby determine its genotoxicity.

Human metabolic polymorphisms in the enzymes catalyzing the activation and/or detoxification pathways of carcinogen metabolism may account for differences in susceptibility to carcinogens between individuals (19–21). For example, CYP1A2 and NAT2 are polymorphically distributed in humans and differences in the activities of these enzymes are postulated to be risk factors for cancer on exposure to HAAs (19, 22). In addition, the enzymes of detoxification, including the UDP-glucuronosyltransferases, display wide heterogeneity in humans. Variables including gender, age, certain disease states, ethnicity, and diet can also modulate the activity of these enzymes (23). Thus, individual variability in the activities of CYP1A2 and NAT2, as well as differences in the UDP-glucuronosyltransferases, may be crucial determinants in the bioactivation and detoxification of HAAs in humans.

Among the HAAs, MeIQx is one of the most abundant and mutagenic formed in cooked meats (1, 2). Dietary studies conducted in humans have provided information on the bioavailability and disposition of MeIQx (24–27). In addition, the metabolic factors affecting the biotransformation of MeIQx have been emphasized. In one study, it was shown that the amounts of MeIQx recovered in the urine of subjects who were treated with the CYP1A2 enzyme inhibitor furafyl-line increased by 14-fold when compared with untreated subjects (28). Recently, it was found that the urinary levels of unchanged MeIQx (29) and unchanged plus amine-conjugated MeIQx (26) are inversely related to individual CYP1A2 activity, which confirms that CYP1A2-mediated metabolism of MeIQx is important in humans. In contrast, NAT2 activity showed no association to the excretion levels of MeIQx and its amine-conjugated metabolites in urine.

Analysis of urinary MeIQx metabolites derived via CYP1A2 *N*-oxi-

Received 4/9/99; accepted 8/19/99.

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¹ Work carried out at the Massachusetts Institute of Technology was supported by National Institute of Environmental Health Sciences Grant ES05622.

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³ Abbreviations used are: HAA, heterocyclic aromatic amine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; [²H₃]MeIQx, 2-amino-3-[²H₃]methyl-8-methylimidazo[4,5-*f*]quinoxaline; *N*-OH-MeIQx, 2-hydroxyamino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; *N*-OH-MeIQx-*N*²-glucuronide, *N*²-(β -1-glucosiduronyl)-2-hydroxyamino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; 2-OH-MeIQx, 2-hydroxy-3,8-dimethylimidazo[4,5-*f*]quinoxaline; 2-nitro-MeIQx, 2-nitro-3,8-dimethylimidazo[4,5-*f*]quinoxaline; CYP1A2, cytochrome P4501A2; NAT2, *N*-acetyltransferase; SIM, selective ion monitor-

ing; HPLC, high-performance liquid chromatography; NCI, negative ion chemical ionization; MS, mass spectrometry; GC, gas chromatography; LC, liquid chromatography.

ation may serve as a biomarker in human risk assessment of this HAA. Although several studies have focused on the determination of MeIQx and its amine-conjugated metabolites in urine, little information is available on the urinary excretion of the *N*-oxidized metabolites of MeIQx, nor has the relationship of CYP1A2 or NAT2 activity to the excretion of these metabolites been described. Recently, the *N*²-glucuronide conjugate of 2-hydroxyamino-MeIQx, *N*-OH-MeIQx-*N*²-glucuronide (Fig. 1), was identified in the urine of five patients administered an oral dose of ¹⁴C-MeIQx (30). To obtain information on the extent of this metabolic pathway in healthy individuals ingesting a uniform meat-based diet, we determined the urinary excretion levels of *N*-OH-MeIQx-*N*²-glucuronide in these subjects. In addition, we examined the role of the enzyme activities of CYP1A2 and NAT2 in the biotransformation of MeIQx to *N*-OH-MeIQx-*N*²-glucuronide. Quantification of the amount of this metabolite in urine will allow an assessment of the *in vivo* capacity of humans to *N*-oxidize MeIQx as well as establish a basis for the role of *N*²-glucuronidation in the detoxification of *N*-OH-MeIQx.

MATERIALS AND METHODS

Chemicals and Reagents. MeIQx, [2-¹⁴C]MeIQx, and [²H₃]methyl-MeIQx were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Isotopic purity of the deuterium-labeled standard was determined by GC-MS to be 96.5%; the *d*₀ contribution was 2.4%. Certify II (a mixed-mode packing composed of C-8 and SAX bonded-phase material) Bond-Elut cartridges (500-mg size) were purchased from Varian (Harbor City, CA). Diisopropylethylamine and 3,5-*bis*(trifluoromethyl)benzyl bromide were obtained from Aldrich Chemical Co. (Milwaukee, WI). The high-purity water used in the procedures was glass-distilled, and the organic solvents (Omnisolve grade) were obtained from EM Science (Gibbstown, NJ). All of the glassware used in the analyses was silanized with a 5% solution of dimethyldichlorosilane in toluene and rinsed with toluene and methanol before use.

Reference Compounds. 2-Nitro-MeIQx was synthesized by the method of Grivas (31). *N*-OH-MeIQx was prepared by the reduction of 2-nitro-MeIQx with ascorbic acid in 29.9% NH₄OH as described previously (9). *N*-OH-MeIQx-*N*²-glucuronide and the related [2-¹⁴C]MeIQx and [²H₃]MeIQx analogues were biosynthetically prepared from the corresponding 2-hydroxyamino-MeIQx derivatives using rat liver S-9 homogenates (32). The incubation mixture consisted of 5 ml of 100 mM KH₂PO₄ (pH 7.6) containing 5 mM MgCl₂, 5 mM glucose-6-phosphate, 6 mM uridine 5'-diphosphoglucuronic acid, 5 units glucose-6-phosphate dehydrogenase, 25 mg of liver S-9 from male rats pretreated with Aroclor, and 150 μg of 2-hydroxyamino-MeIQx. The metabolites were purified and characterized under the conditions described previously (33).

2-OH-MeIQx was prepared synthetically by treating 2-nitro-MeIQx (0.5 mg) with 0.4 ml of 1.0 N NaOH and heating for 2 h at 37°C. After neutralization of the reaction mixture with 0.4 ml of 1 N HCl, the sample was applied to a C-18 Sep-Pak. The cartridge was washed once with 1 ml of water to remove salts, and 2-OH-MeIQx was eluted with 2 ml of methanol. The sample

was analyzed by HPLC using a 5-μm particle size C₁₈ column (25 cm × 4.6 mm, Supelco, Inc., Bellefonte, PA) with a mobile phase consisting of 50 mM (pH 6.8) ammonium acetate:methanol (95:5, by vol.) for 5 min, followed by a linear gradient to 100% methanol over 30 min at a flow rate of 1 ml/min. HPLC analysis of the product derived from base treatment of 2-nitro-MeIQx showed a single major peak; the purity of the compound exceeded 90% by HPLC. The synthetic product eluted at a retention time of 21 min; the on-line HPLC-UV spectrum had an absorption maximum at 267 nm. The hydrolysis product was analyzed by LC-MS in positive ion mode, and the full scan mass spectrum exhibited a protonated molecular ion at *m/z* 215. This is consistent with a structure in which the 2-nitro group has been replaced by a hydroxyl group to give 2-OH-MeIQx. With a smaller-scale preparation, 2-OH-MeIQx was prepared by heating *N*-OH-MeIQx-*N*²-glucuronide (5 μg) in 0.5 ml of a solution composed of glacial acetic acid:acetonitrile:H₂O (10:10:1, by vol.) at 60°C for 6 h (Fig. 1). After evaporation of the solvent under N₂, the reaction product was analyzed by HPLC with UV detection using the conditions described above. Quantitative conversion to a single major product was evident with no intact conjugate present. The compound was identified by online UV spectra analysis and comigration with the synthetic 2-OH-MeIQx prepared from base treatment of 2-nitro-MeIQx (described above). 2-OH-MeIQx was further characterized by both electron ionization GC-MS and NCI-GC-MS after derivatization with 3,5-*bis*(trifluoromethyl)benzyl bromide (25). A major chromatographic peak was obtained by GC-MS analysis. The full scan mass spectrum showed a molecular cation (or molecular anion under NCI-GC-MS conditions) at *m/z* 440. This corresponds to a derivative of 2-OH-MeIQx containing a single 3,5-*bis*(trifluoromethyl)benzyl group.

Study Design. More detailed accounts of the study design and the participating subjects have been published elsewhere (29, 34). Briefly, the individuals were recruited from the Beltsville, MD area. The enrollment criteria included: (a) being in good health; (b) being a nonsmoker for at least 6 months; (c) having weight not less than 90% or greater than 130% of 1983 Metropolitan Life Insurance desirable weights; (d) not consuming any atypical diet; (e) taking no medication other than an occasional analgesic; and (f) being able to consume caffeine.

Meat Preparation and Controlled Dietary Period. The meat preparation and dietary protocol have been reported previously (29, 34). In brief, the subjects consumed a controlled diet containing differing amounts of meat based on their body weight (3.1–4.0 g meat/kg body weight). The well-done cooked beef contained 9 ng/g of MeIQx. Urine collections were made 0–12 and 12–24 h after consumption of the meal. These collections (obtained in a prior study dating from 1993) were stored frozen at –80°C until thawed, and aliquots were prepared for subsequent analysis. Animal studies have found that urines stored at –80°C for prolonged periods of time (>1 year) exhibited no noticeable change in the *N*-OH-MeIQx-*N*²-glucuronide metabolite based on ¹⁴C-HPLC analysis.

CYP1A2 and NAT2 Phenotype. The subjects were phenotyped for CYP1A2 and NAT2 function by measuring the ratio of urinary caffeine metabolites by the procedure of Butler *et al.* (35). CYP1A2 was determined using the molar urinary ratio of 1,7-dimethylxanthine-plus-1,7-dimethyluracil:1,3,7-trimethylxanthine. NAT2 was calculated using the molar urinary ratio of 5-acetyl-amino-6-formylamino-3-methyluracil:1-methylxanthine. These analyses, carried out at the National Center for Toxicological Research, have been described previously (29, 34).

Immunoaffinity Chromatography. Monoclonal antibodies raised against MeIQx (36) were used in the preparation of immunoaffinity columns. The antibodies were immobilized on CNBr-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO) at a concentration of 4 mg of protein/ml of gel. The antibody gel preparations were washed with 50% DMSO in water (10 bed volumes), followed by an equal volume of methanol:water (90:10) and then water. The antibody-bound gels were stored in PBS containing 4 mM sodium azide at 4°C. Small cartridges of the gel were prepared using 2 ml of gel suspension, and the binding efficiency of the purified *N*-OH-MeIQx-*N*²-glucuronide standard was determined by the following procedure. On rinsing the gel suspension with 15 bed volumes of ammonium acetate [50 mM (pH 5.5)], the sample compound dissolved in 0.5 ml of ammonium acetate [50 mM (pH 5.5)] was applied to the cartridge. The gel was washed with 10 bed volumes of ammonium acetate buffer [50 mM (pH 5.5)] followed with 20 bed volumes of water. The standard compound was eluted from the gel with 7 bed volumes of methanol:water (90:10). The eluant was dried under vacuum using a centrifugal evaporator (Savant Instruments, Farmingdale, NY). Fractions were ana-

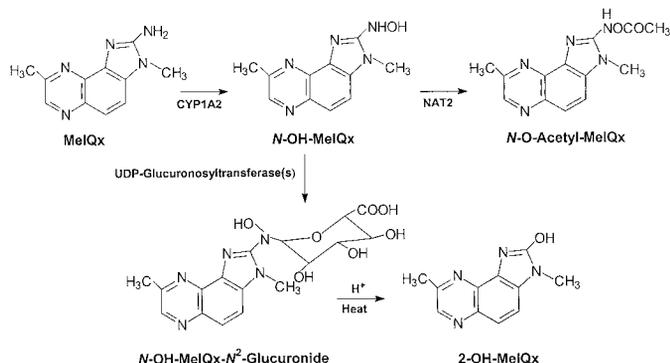


Fig. 1. Simplified scheme of the pathways of MeIQx metabolism leading to the formation of *N*-oxidation products. The conversion of *N*-OH-MeIQx-*N*²-glucuronide under acidic conditions to form 2-OH-MeIQx is illustrated.

lyzed for the conjugate by UV detection, and the binding efficiency of the antibody-modified gel was determined to be 90–95%.

Isolation of Urinary *N*-OH-MeIQx-*N*²-Glucuronide. *N*-OH-MeIQx-*N*²-glucuronide was isolated from urine by a two-step procedure involving an initial solid-phase extraction followed by an immunoaffinity purification of the resulting extract. In the procedure, Certify II solid-phase extraction cartridges were washed and conditioned before use with 40 ml of methanol, then with 25 ml of water, followed by 25 ml of 50 mM ammonium acetate buffer (pH 7.1). Stock solutions of the internal standard [²H₃]methyl-*N*-OH-MeIQx-*N*²-glucuronide were prepared in methanol and quantified with a Hewlett-Packard 8452A spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) using an extinction coefficient value of 38,900 M⁻¹cm⁻¹ for the standard at 274 nm (33). Urine samples (typically 8 ml), diluted with 4 ml of 0.1 M ammonium acetate buffer (pH 7.1), were spiked with 6.5 ng of [²H₃]methyl-*N*-OH-MeIQx-*N*²-glucuronide and applied to the preconditioned Certify II cartridges by gravity flow. The cartridges were washed successively with 15 ml of 50 mM ammonium acetate buffer (pH 7.1), 10 ml of water, and then with 1 ml of methanol. The bound *N*-OH-MeIQx-*N*²-glucuronide was eluted with 14 ml of methanol:1 N acetic acid (80:20, by vol.). After neutralizing the eluants with the addition of 0.1–0.2 ml of 29.9% ammonium hydroxide (final pH 6.5–7.0), the solutions were purged with N₂ for 0.5 min and then evaporated to near dryness by vacuum centrifugation. Immunoaffinity columns using MeIQx monoclonal antibody gel preparations were used to isolate *N*-OH-MeIQx-*N*²-glucuronide-MeIQx and its deuterated internal standard in the urine extracts. On addition of 5 ml of 50 mM ammonium acetate (pH 5.5) to the extracts, the samples were applied to the prewashed and conditioned antibody gel preparations (described above). The eluants were collected and reappplied to the gels a second time. The gels were washed with 20 ml of 50-mM ammonium acetate (pH 5.5), followed by 40 ml of water. The bound-conjugate fraction was eluted with 14 ml of methanol:water (90:10) and dried by vacuum centrifugation. The dried samples were then transferred with methanol (3 × 0.3 ml) to Reactivials. After evaporation of the methanol by a stream of N₂, the samples were treated with 100 μl of a solution of glacial acetic acid:acetonitrile:water (50:45:5, by vol) and heated at 75°C for 2 h. After cooling, the acidic solutions were evaporated to dryness under a stream of N₂. After the addition of 0.5 ml of 0.5 M Na₂CO₃ solution to the dried samples, 2-OH-MeIQx was extracted using ethyl acetate (2 × 0.5 ml). The ethyl acetate layers were transferred to small Reactivials and evaporated under N₂. Derivatization of the samples was carried out by a previously described procedure using 3,5-bis(trifluoromethyl)benzyl bromide and diisopropylamine (25). After removal of the derivatizing reagents by evaporation with N₂, 0.2 ml of 0.5 M Na₂CO₃ was added, and the solutions were extracted 3 times with 0.5 ml of hexane. The hexane extracts were combined and evaporated under N₂ and then reconstituted in 25 μl of hexane for subsequent GC-MS analysis.

NCI-GC-MS Analysis. The derivatized samples were analyzed using a Hewlett-Packard 5989A GC-MS equipped with a standard electron ionization/chemical ionization source with positive and negative ion detection. The instrument was manually tuned for maximum sensitivity in the ionization mode to be used. For negative ion chemical ionization, with methane as the moderating gas, the source pressure was typically 2.0 Torr, the electron energy was 200 eV, the source temperature was 220°C, and the emission current was 350 uA. The gas chromatographic column was a 15 m × 0.25 mm DB-35 ms fused-silica capillary (J & W Scientific, Inc., Rancho Cordova, CA). Helium was the carrier gas, and a flow rate of approximately 2 ml/min was used. Samples were injected in the splitless mode with an initial oven temperature of 150°C, followed by a temperature ramp to 300°C at 20°C/min. For analysis of urine extracts, the mass spectrometer was operated in the selective ion monitoring mode using the negative ions at *m/z* 440 for 2-OH-MeIQx and *m/z* 443 for its deuterated analogue. Quantification of the analyte in the extracts was performed using the peak areas as determined by the integration routine in the data system. Standards were analyzed under the same conditions and used to correct for the background contribution of nondeuterated 2-OH-MeIQx in the isotopically labeled 2-OH-MeIQx. Control urine samples obtained from individuals who consumed no meat 24 h before collection were analyzed to determine the presence of background in the assay.

Recovery Studies. The overall recovery of 2-OH-MeIQx derived from *N*-OH-MeIQx-*N*²-glucuronide in urine was determined by spiking urine samples with known amounts of ¹⁴C-labeled *N*-OH-MeIQx-*N*²-glucuronide. Urine samples were carried through the entire procedure described above and the amounts of radiolabeled 2-OH-MeIQx in the derivatized extracts were assessed by scintillation counting.

Precision and Accuracy of the Assay. Replicate analyses of aliquots of urine from a pooled sample were made during the course of the study to determine the precision of the assay.

The accuracy of the method was assessed by determining nanograms of *N*-OH-MeIQx-*N*²-glucuronide recovered *versus* nanograms of *N*-OH-MeIQx-*N*²-glucuronide added before extraction. Analyses were carried out using control urine samples (in duplicate) spiked with increasing amounts (0–17 ng) of *N*-OH-MeIQx-*N*²-glucuronide in the presence of a constant amount (6.5 ng) of the [²H₃]methyl analogue. After isolation and treatment of the conjugate fraction as described above, 2-OH-MeIQx and its [²H₃]methyl analogue were derivatized and quantified by NCI-GC-MS SIM analysis.

Calibration curves were generated for 2-OH-MeIQx by adding increasing amounts of *N*-OH-MeIQx-*N*²-glucuronide (0–22 ng) in the presence of a constant amount (8 ng) of the stable isotope-labeled internal standard. After acid-hydrolysis, the standard compounds were extracted, derivatized, and analyzed by SIM. Plots of the peak-area ratio of analyte:internal standard of 2-OH-MeIQx against the ratio of amount in ng of analyte:internal standard were made.

Statistical Analysis. Linear regression analysis was performed to determine the relationship between the excretion level of *N*-OH-MeIQx-*N*²-glucuronide in urine *versus* the ingested dose of MeIQx. In addition, Spearman rank-correlation tests were used to determine associations between the data sets. Differences in means were analyzed by Student's *t* test. To determine whether the excreted amount of *N*-OH-MeIQx-*N*²-glucuronide in urine, controlled for the amount of meat eaten, was related to interindividual variation in the activity levels of CYP1A2 and NAT2, we performed a multiple regression analysis of total *N*-OH-MeIQx-*N*²-glucuronide *versus* the levels of these enzymes.

RESULTS

Standard Measurements and Urinary Analysis. The technique using Certify II solid-phase extraction combined with immunoaffinity separation was found to be very specific for the isolation of *N*-OH-MeIQx-*N*²-glucuronide from urine. The selectivity of the monoclonal antibody assay ensured a highly purified extract containing *N*-OH-MeIQx-*N*²-glucuronide. 2-OH-MeIQx (if it is present in urine before acid treatment) would not be isolated by this procedure inasmuch as this compound does not bind to the Certify II cartridge nor to the monoclonal antibody. Furthermore, to our knowledge, none of the other MeIQx metabolites that are thus far identified in rodents or nonhuman primates can be converted into 2-OH-MeIQx.

The overall recovery of 2-OH-MeIQx after the isolation and hydrolysis of urinary *N*-OH-MeIQx-*N*²-glucuronide was estimated to be, on the average, 55 ± 9% (±SD) based on spiking studies with the ¹⁴C-labeled compound.

Calibration plots, constructed from the SIM analysis of derivatized 2-OH-MeIQx, *versus* its [²H₃]analogue, formed from the corresponding *N*-OH-MeIQx-*N*²-glucuronides exhibited linearity over the range of 0–22 ng/sample (*r*² = 0.99; slope = 0.96; intercept = 0.05).

The precision of the assay procedure was assessed by analysis of replicate samples (*n* = 8) over a period of 4 months. These measurements gave a level of 472 ± 40 pg/ml (mean ±SD) of *N*-OH-MeIQx-*N*²-glucuronide in urine assayed as 2-OH-MeIQx by NCI-GC-MS SIM; the intersample coefficient of variation was 8.5%. In addition, the accuracy of the assay was validated by determining nanograms of *N*-OH-MeIQx-*N*²-glucuronide recovered *versus* nanograms of *N*-OH-MeIQx-*N*²-glucuronide added before sample work-up. The accuracy for spiked samples (recovery) fell between 91 and 97%.

Analysis of urine samples obtained from subjects who did not eat meat within a 24-h period demonstrated no detectable presence of *N*-OH-MeIQx-*N*²-glucuronide (inset panel, Fig. 2). Good detection limits in the low picogram range for derivatized 2-OH-MeIQx injected on column were observed with no background interference. This method allowed detection of *N*-OH-MeIQx-*N*²-glucuronide at a lower limit of 80 pg of analyte in 8 ml of urine assuming a signal to noise ratio of 3 to 1. Fig. 2 illustrates a NCI-GC-MS SIM analysis of 2-OH-MeIQx derived from

N-OH-MeIQx-*N*²-glucuronide in a urine sample; the concentration of *N*-OH-MeIQx-*N*²-glucuronide in the urine was 415 pg/ml.

Subject Characteristics and Excretion of *N*-OH-MeIQx-*N*²-Glucuronide. The subject characteristics, the quantity of meat eaten, and the range and median values for CYP1A2 and NAT2 activities have been presented previously (26, 29, 34). Briefly, there were 66 subjects (33 males and 33 females) enrolled in the study; their ages ranged from 27 to 62 years. The total amount of high-temperature cooked beef eaten by each individual ranged from 180 to 328 g, with a median value of 248 g. The values for CYP1A2 activity within the individuals ranged from 2.1 to 28, whereas the NAT2 activity ranged from 0.19 to 3.7.

The values for the mean, median, and range of the amount of *N*-OH-MeIQx-*N*²-glucuronide in the 0–12 h urine are presented in Table 1. The amounts of *N*-OH-MeIQx-*N*²-glucuronide recovered in the 0–12 h urine, for all of the subjects, ranged from 78 to 915 ng, with an average value of 391 ± 138 ng (\pm SD). The excretion level of *N*-OH-MeIQx-*N*²-glucuronide in the 0–12 h urine ranged from 2.2 to 17.1% of the ingested dose, with an average value of $9.4 \pm 3.0\%$ (\pm SD).

A positive relationship was found (Fig. 3) between the amount of *N*-OH-MeIQx-*N*²-glucuronide excreted in urine and the amount of MeIQx ingested by each subject ($r^2 = 0.24$; $P < 0.0001$). The same dataset analyzed using Spearman's rank correlation showed a strong correlation ($r_s = 0.44$; $P = 0.0002$).

We also examined the possible associations between the urinary excretion of *N*-OH-MeIQx-*N*²-glucuronide and other individual characteristics, such as the age and sex of the subjects. A comparison of the mean values of *N*-OH-MeIQx-*N*²-glucuronide excretion (adjusted for meat intake) by the males *versus* the females at the 95% confidence level showed no difference ($P = 0.22$). No association was seen between the age of the subjects and the normalized amount of *N*-OH-MeIQx-*N*²-glucuronide in urine ($r^2 = 0.04$; $P = 0.11$).

Comparison (Fig. 4) of the urinary levels of *N*-OH-MeIQx-*N*²-glucuronide *versus* combined MeIQx (unchanged MeIQx plus amine-conjugated MeIQx), values obtained in the previous investigation

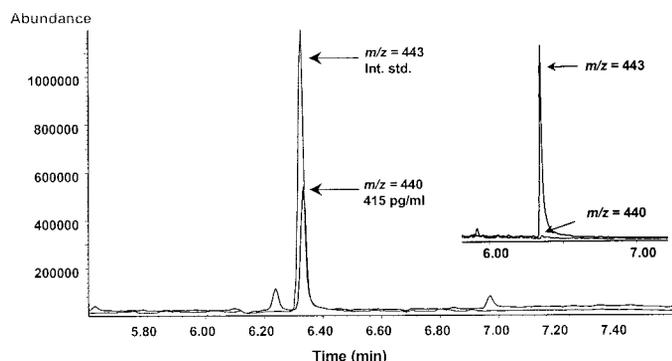


Fig. 2. NCI-GC-MS selected ion chromatograms obtained after the isolation and analysis of urinary *N*-OH-MeIQx-*N*²-glucuronide to form 2-OH-MeIQx. 2-OH-MeIQx was analyzed as the 3,5-*bis*(trifluoromethyl)benzyl derivative; the negative ions at *m/z* 440 and 443 arise from 2-OH-MeIQx and the *d*₅-methyl standard, respectively. The concentration of *N*-OH-MeIQx-*N*²-glucuronide in the subject's sample was 415 pg/ml. The inset in the figure depicts a control urine sample analyzed under the same conditions.

Table 1 Urinary excretion level of *N*-OH-MeIQx-*N*²-glucuronide

	Mean \pm SD	Median	Range
<i>N</i> -OH-MeIQx- <i>N</i> ² -glucuronide, ng in 0–12 h urine	391 ± 138	408	78–915
<i>N</i> -OH-MeIQx- <i>N</i> ² -glucuronide, % of dose in 0–12 h urine	9.4 ± 3.0	9.5	2.2–17.1

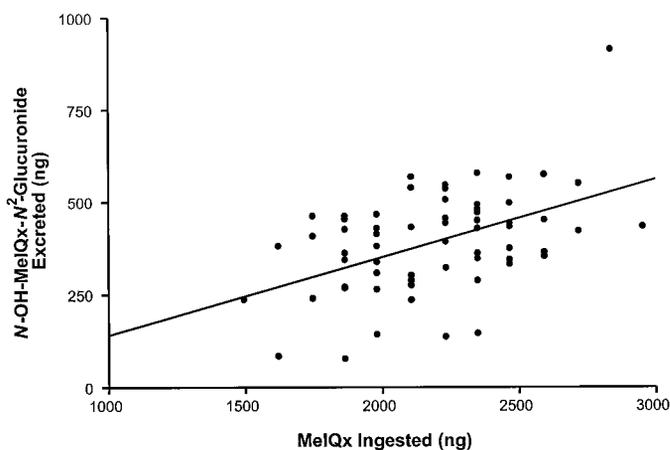


Fig. 3. Correlation of the total amount in ng of *N*-OH-MeIQx-*N*²-glucuronide excreted in the 12-h urine as a function of the amount of MeIQx ingested for each subject.

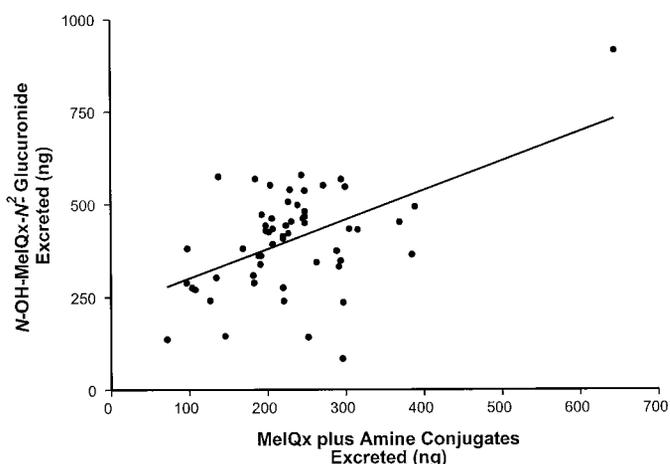


Fig. 4. Relationship between the excretion levels of *N*-OH-MeIQx-*N*²-glucuronide and MeIQx (unchanged plus amine-conjugated MeIQx) in urine, measurements reported earlier (26).

(26), showed a correlation of $r_s = 0.33$ ($P = 0.01$). The correlation with the one high-value (outlier) removed was $r_s = 0.29$; $P = 0.03$.

CYP1A2 and NAT2 Phenotype *versus* Excretion Level of *N*-OH-MeIQx-*N*²-Glucuronide. The subjects were phenotyped for CYP1A2 and NAT2 activities in the period before the consumption of the high temperature-cooked beef (29). To determine whether the amount of *N*-OH-MeIQx-*N*²-glucuronide excreted was associated with interindividual variation in CYP1A2 and NAT2 phenotypes, we evaluated the effect of these enzymes in a regression that controlled for the amount of meat ingested. To increase the accuracy and efficiency of the statistical method and to decrease the sensitivity of the analysis to individual points, two standard transformations were used: a \log_{10} transformation was applied to CYP1A2 and NAT2; and a square root transformation was applied to urinary *N*-OH-MeIQx-*N*²-glucuronide. No association was found between activity levels of CYP1A2 or NAT2 and the urinary excretion of *N*-OH-MeIQx-*N*²-glucuronide in the subjects ($P = 0.65$ and 0.25 , respectively).

DISCUSSION

Previous investigations carried out within this study group of healthy individuals ingesting a uniform meat-based diet have described the excretion levels of MeIQx and its amine-conjugated metabolites in urine and their relation to CYP1A2 and NAT2 activity. In

this study, we determined the interindividual urinary excretion level of the N^2 -glucuronide conjugate of N -OH-MeIQx and its relation to CYP1A2 and NAT2 activity. We found that this metabolite is readily detected in urine after ingestion of MeIQx in the diet and that the amount excreted correlates with the ingested dose. The individuals excreted a range of 2.2–17.1% (median, 9.5%) of the ingested dose of MeIQx as N -OH-MeIQx- N^2 -glucuronide in urine, indicating the extent and variability in the excretion level of this conjugate. No association was found between the level of N -OH-MeIQx- N^2 -glucuronide in urine and the enzymic activity of CYP1A2. These results may be explained in part by the evidence obtained in a prior investigation (28), in which the elimination of MeIQx was found to be first order and very rapid with a half-life of <4 h; moreover, CYP1A2-catalyzed metabolism accounted for ~91% of the elimination of ingested MeIQx. The subjects in our study ingested very low doses of MeIQx (a median of 2.2 μ g per person; range, 1.6–3.0 μ g). Because the metabolism of MeIQx in humans is rapid and first order at the doses given, we would not expect to see an effect of CYP1A2 activity on the urinary excretion of a secondary product derived from the primary oxidation product. That is, the amount of N -OH-MeIQx- N^2 -glucuronide formed would depend primarily on the efficiency of the enzyme(s) involved in the glucuronidation of the N -hydroxylamine metabolite and not on the activity level of CYP1A2. In addition to this conjugation pathway, recent work has established that NADH-dependent reduction of N -hydroxyarylamines to the parent amines occurs in human liver microsomes (37). The involvement of this process would limit the bioavailability of the N -hydroxylamine metabolite to undergo subsequent conjugation. Furthermore, divergent pathways of metabolism such as the phase II sulfotransferase and glucuronosyltransferase(s) reactions compete with CYP1A2 in reactions with the parent amine. These conjugation reactions can influence the amount of the parent amine available for N -oxidation. The previous findings of an inverse association between CYP1A2 activity and the urinary levels of MeIQx (29) and MeIQx plus its amine conjugates (26) among this same group of study subjects reflect pathways unrelated to the formation of N -OH-MeIQx- N^2 -glucuronide. CYP1A2 is known to be involved also in the formation of 2-amino-8-(hydroxymethyl)-3-methylimidazo[4,5-*f*]quinoxaline; this metabolite accounts for 1.0–4.4% of the dose in humans (30). Between 20 and 58% of the dose of MeIQx is excreted in urine (30); however, the major urinary metabolite of MeIQx in humans remains unidentified at present.

A correlation was observed between the urinary excretion levels of N -OH-MeIQx- N^2 -glucuronide and MeIQx plus its amine-conjugated metabolites, values determined in an earlier study (26), which suggests that the clearance of these metabolic products is similar within each subject. The levels of these metabolic products are both positively related to the amount of MeIQx consumed and may accordingly be used as biomarkers of dietary exposure to this HAA.

We did not observe an inverse association between the amount of N -OH-MeIQx- N^2 -glucuronide in urine and the activity level of NAT2 within the subjects. It has been demonstrated in previous work that MeIQx is not N -acetylated by human liver cytosol (9) nor by human NAT2 expressed in *COS*-1 cells (11). However, N -OH-MeIQx is converted to a highly reactive N -acetoxyarylamine by reaction with human liver and colon cytosol *O*-acetyltransferase (9) and recombinant human NAT2 expressed in *COS*-1 cells (11). Among the acetyltransferases, NAT2 is more important than NAT1 for the bioactivation of most of the N -OH-HAAs (11, 19). In humans, NAT2 exhibits a polymorphism that has allowed individuals to be designated as phenotypically slow or rapid acetylators. Slow-acetylator individuals have genetic mutations that result in a less functional NAT2 enzyme (19). In human liver cytosol isolated from slow acetylators, the rate of *O*-acetylation of N -OH-HAAs was generally about one-third of that observed for the rapid acetylator prep-

arations (9). In addition to the N -*O*-acetyltransferases, other enzymes, including the thermostable sulfotransferases, may contribute to the further metabolism of N -OH-MeIQx to highly reactive intermediates (12). Therefore, metabolic reactions via sulfotransferases and N -*O*-acetyltransferase could, in principle, compete with the N^2 -glucuronidation of N -OH-MeIQx. Furthermore, interindividual differences in the N^2 -glucuronosyltransferase(s) reactions may directly influence the rates of formation of N -OH-MeIQx- N^2 -glucuronide with respect to the other pathways. Thus, if competing pathways of metabolism are present, the distribution of the metabolic products will depend on the relative activities of the different enzymes. Further work is needed to characterize the relative activities and substrate specificities for the conjugation reactions of N -OH-MeIQx. Our results show that N^2 -glucuronidation of N -OH-MeIQx is an important detoxification pathway leading to the formation of a more polar and excretable metabolite, thereby playing an essential role in the elimination of N -OH-MeIQx from the body.

There are considerable differences between humans and animal species in the levels of N -OH-MeIQx- N^2 -glucuronide recovered in urine. The amounts in human urine are on average 10-fold higher than those found for rodents (32) and nonhuman primates (38), which indicates that CYP1A2-mediated N -oxidation of MeIQx and/or N^2 -glucuronide conjugation reactions of the N -hydroxylamine metabolite via UDP-glucuronosyltransferases are higher in humans than are those for the other species. *In vitro* studies (7) have demonstrated that human liver samples, in some cases, are as active as liver samples from rats pretreated with potent CYP1A2 inducers in activating MeIQx to mutagenic metabolites. Furthermore, higher rates of N^2 -glucuronidation of N -OH-MeIQx have been observed with human microsomes than with rat microsomes (14). A combination of these factors may, thus, determine the relative amount of N -OH-MeIQx- N^2 -glucuronide formed in humans with respect to the other species; however, interspecies differences in excretion pathways cannot be excluded. In the present study, the level of N -OH-MeIQx- N^2 -glucuronide in urine varied considerably among the subjects, which indicated that interindividual differences in the activity or specificity of the UDP-glucuronosyltransferases may be important in the disposition of N -OH-MeIQx in humans, although interindividual variability in elimination pathways must be considered.

It is not known which of the human UDP-glucuronosyltransferase isozymes is responsible for the glucuronidation of the N -hydroxylamine metabolites of the HAAs; however, the family of isozymes making up the UDP-glucuronosyltransferases exhibits a wide diversity in enzyme activities, and the activities seem to be inducible in humans (19). The extent to which this variability is a risk factor in HAA genotoxicity *in vivo* remains to be clarified.

In the present work, we developed a procedure that provided a sensitive and specific method for determining the amount of N -OH-MeIQx- N^2 -glucuronide excreted in urine. The selectivity of the monoclonal antibody assay assured a facile method by which to free urine extracts of interference from related components and background material. Acidic hydrolysis of N -OH-MeIQx- N^2 -glucuronide to form 2-OH-MeIQx and subsequent analysis of the derivatized product by NCI-GC-MS is an effective method for the determination of a polar semistable metabolite in urine. Other HAAs, including PhIP, may be expected to undergo similar pathways of metabolic activation and detoxification. Determination of the urinary excretion levels of these N -oxidized conjugates will be a subject of interest in future human biomonitoring studies.

To ascertain the health risks of HAAs in humans, various approaches have been used, including the analysis of urinary HAAs, identification of urinary metabolites, and association of these determinants with individual metabolic phenotypes. Monitoring urinary HAA excretion has been essential in evaluating HAA exposure in humans ingesting unrestricted diets (39–41). In addition, these pro-

cedures are valuable in assessing the effect of dietary intervention in enhancing the levels of conjugated or detoxified HAA metabolites in urine (42). In this study we examined the role of conjugation in the detoxification of *N*-OH-MeIQx in humans by analysis and quantification of its *N*²-glucuronide conjugate in urine. Our results demonstrate that MeIQx is readily metabolized *in vivo* to form this conjugate and that it is excreted in urine in a dose-related manner. Monitoring the urinary excretion level of *N*-OH-MeIQx-*N*²-glucuronide serves as an indicator of the bioactivation and detoxification pathways of MeIQx metabolism in humans. Further work in defining the role of the UDP-glucuronosyltransferase(s) in the detoxification of *N*-OH-MeIQx and other *N*-OH-HAAs may be necessary as part of the risk assessment process.

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