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N-Demethylation is a major route of 2-amino-3-methylimidazo[4,5-*f*]quinoline

metabolism in mouse

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Non-standard abbreviations: IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; HCA, heterocyclic

amine; DSS, dextran sulfate sodium; P450, cytochrome P450; Demethyl-IQ, 2-amino-imidazo[4,5-

f]quinoline; DETAPAC, diethylenetriaminepentaacetic acid; EROD, 7-ethoxyresorufin *O*-

deethylation; N-acetyl-IQ, N-acetyl-2-amino-3-methylimidazo[4,5-*f*]quinoline; MS, mass

spectrometry; CAD, collisionally activated dissociation; ESI, electrospray ionization;

Abstract

2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) metabolism was evaluated in mouse to better understand its tumorigenicity. Urinary metabolites from mice orally administered 40 mg/kg ^{14}C-IQ were compared to similarly treated rats. The recovery of radioactivity was significantly greater in mouse urine. The relative proportion of metabolites was significantly different and a new rodent metabolite detected. For rat, the proportion of previously identified metabolites excreted was 5-O-glucuronide > sulfamate > 5-sulfate > N-glucuronide. In mouse urine, a new metabolite, Demethyl-IQ, represented about 26% of IQ metabolism with the proportion of metabolites the following: 5-O-glucuronide > Demethyl-IQ > sulfamate > N-glucuronide > 5-sulfate. Mouse metabolites were identified by electrospray ionization mass spectrometry. Demethyl-IQ was shown to be 2-amino-imidazo[4,5-*f*]quinoline. N-Acetyl-2-amino-3-methylimidazo[4,5-*f*]quinoline was not detected with mice. Mouse liver slices produced 5-O-glucuronide, Demethyl-IQ, and sulfamate with the former two significantly reduced by ellipticine. Liver microsomes only produced Demethyl-IQ. Ellipticine, P450 1A inhibitor, but not furafylline, 1A2 selective inhibitor, prevented microsomal N-demethylation. Inhibitors had similar effects on EROD activity. Demethyl-IQ was not further metabolized by an intact mouse or liver microsomes. Thus, mouse IQ metabolism is significantly different from rat and these differences may affect IQ tumorigenicity. N-Demethylation of IQ-like HCAs occurs in mouse, monkey and human, but not rat.

Introduction

2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) is a heterocyclic amine (HCA) formed by high temperature cooking of proteinaceous food (Felton and Knize, 1990). More than 20 similarly formed HCAs have been identified. In the human diet, consumption of 400 gm of cooked lean meat could result in exposure to several micrograms of mutagenic HCAs. These amines are detected in urine and their excretion, as unchanged amine, increased by a cytochrome P450 (P450) inhibitor (Lynch et al., 1992;Boobis et al., 1994), indicating their absorption from cooked foods and P450 metabolism.

IQ is a potent carcinogen inducing tumors in multiple tissues and in different species (NTP, 2005). In mouse, oral administration of IQ caused forestomach, liver, and lung tumors in both sexes. While in rat IQ also caused tumors in several organs in both sexes, mammary and clitoral gland tumors were only detected in females and colon and skin tumors in males. IQ administered orally to monkeys or mice caused liver tumors. Using a colitis-induced carcinogenicity model with Apc$^{Min/+}$ mice (Cooper et al., 2001), we have preliminary data indicating that induction of colitis by treatment with 4% dextran sulfate sodium (DSS) in drinking water followed by oral doses of 40 mg/kg IQ resulted in a 100% incidence of invasive colorectal tumors, while mice treated with only DSS had no tumors (Clapper et al., 2006). The strong association of high temperature cooked meat intake and colorectal cancer risk (Giovannucci et al., 1994), the presence of HCAs in cooked meat (Felton and Knize, 1990), and the initiation of colon cancer by HCAs (Kristiansen et al., 1997), suggest that HCAs present in high temperature cooked meat may be responsible for the increased risk of colon cancer associated with this dietary component.

Activation of IQ is thought to involve N-oxidation by P450, followed by O-acetylation with subsequent formation of a reactive intermediate, nitrenium ion, that binds DNA (Turesky et al., 1991). These DNA adducts are thought to cause mutations that initiate carcinogenesis. Following

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IQ administration, DNA adducts are found in a variety of rodent and non-human primate tissues (Nerurkar et al., 1995;Turesky et al., 1996). While N-oxidation of IQ has been demonstrated with rat microsomes (Turesky et al., 1998), excretion of N-hydroxy-IQ has not been detected in rat. There is a considerable amount of evidence that P450s play an important role in HCA genotoxicity.

The major pathway for IQ inactivation involves P450 catalyzed ring oxidation at the 5 position followed by conjugation with sulfate or glucuronic acid (Figure 1) (Luks et al., 1989;Inamasu et al., 1989). The other major route of metabolism is direct conjugation of the exocyclic amine to form N^2-glucuronide or sulfamate (Luks et al., 1989;Inamasu et al., 1989;Turesky et al., 1986). The 5-O-glucuronide and 5-sulfate were detected in urine and bile, but not in feces (Luks et al., 1989;Inamasu et al., 1989). In contrast, sulfamate excretion was observed in urine, bile, and feces (Luks et al., 1989;Inamasu et al., 1989;Turesky et al., 1986). This is consistent with the conjugates being hydrolyzed by bacterial enzymes in the intestine and reabsorbed from the intestinal tract. Similar pathways for activation and inactivation have been reported in different species and for other aminoimidazole HCAs (Snyderwine et al., 1992;Langouet et al., 2001). Rat P450 oxidation appears to favor the 5 position, while in humans N-oxidation may be favored (Turesky et al., 1998). Metabolism by the monkey is different from rat in that it forms 2-amino-imidazo[4,5-*f*]quinoline (Demethyl-IQ) as a major product (Snyderwine et al., 1992). Human hepatocytes metabolize 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, a structurally similar HCA, to an N-demethyl derivative, while rat hepatocytes do not (Langouet et al., 2001). Although N-acetylation plays a major role in metabolism of aromatic amines (Lakshmi et al., 1995a), the N-acetyltransferase pathway plays only a minor role in IQ rat metabolism (Inamasu et al., 1989).

Although metabolism can influence tumorigenicity, only balance studies of IQ excretion (Sjodin and Jagerstad, 1984) and its organ distribution (Alldrick and Rowland, 1988;Bergman,

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1985) has been assessed in mouse. Because the mouse is an attractive model for testing

tumorigenicity and its use in this area is likely to continue and even increase, we have evaluated

pathways for mouse IQ metabolism. Results indicate mouse IQ metabolism is similar to monkey

and human, but different from rat. Thus, parameters that affect IQ tumorigenicity in rat and mouse

may be different.

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Methods

Materials. [2-^{14}C]-IQ (10 mCi/mmol, >98% radiochemical purity) and IQ were purchased from

Toronto Research Chemicals (Toronto, ON, Canada). Diethylenetriaminepentaacetic acid

(DETAPAC), acetyl CoA, esterase from porcine liver, dithiothreitol, NADPH, ellipticine,

ethoxyresorufin, resorufin, alpha-naphthoflavone, furafylline, SKF-525A, ketoconazole,

sulfaphenazole, *E. coli* beta-glucuronidase (Type VII-A), and Abalone sulfatase (Type VIII) were

purchased from Sigma-Aldrich Corporation (St. Louis, MO). Ultima-Flo AP was purchased from

PerkinElmer LAS, Inc., Shelton, CT.

Animals and dosing. Six-week old C57Bl/6 female mice were purchased from Jackson

Laboratory, Bar Harbor, ME. Mice were fed AIN-76A from Bio-Serv, Frenchtown, NJ for 8 weeks

before use. Female F344 rats at 13 weeks of age were purchased from Harlan, Indianapolis, IN and

fed the AIN-76A diet for 8 weeks before use. Experiments were performed according to the

National Institutes of Health standards for care and use of experimental animals and the St. Louis

VA Medical Center Animal Care and Use Committee. Animals were group-housed, maintained on

a 12-h light/dark cycle, and had access to food and water *ad libitum*. Mice were administered 40

mg/kg ^{14}C-IQ (20 uCi; 0.2 ml per 20 gm oral dose; 20% DMSO : 80% 0.05 N HCl), placed in

metabolic cages, and a 24-hour urine collected. Rats were administered 40 mg/kg ^{14}C-IQ (20 uCi;

2.5 ml per 250 gm oral dose; 20% DMSO : 80% 0.05 N HCl), placed in metabolic cages, and a 24-

hour urine collected. The recovery of radioactivity in the 24-hour urine from rats and mice was 42

$\pm$ 2% and 55 $\pm$ 3% of the total radioactivity administered, respectively (p < 0.05). The 24- to 48-

hour urinary excretion of radioactivity from rats and mice was 4.2 $\pm$ 0.3% and 3.4 $\pm$ 0.5%,

respectively. To assess a different route for administering IQ, F344 rats were also administered ip

20 mg/kg ^{14}C-IQ (20 uCi; 1.25 ml per 250 gm injected i.p.; 20% DMSO : 80% 0.1 N HCl), placed

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in metabolic cages, and a 24-hour urine collected. The recovery of radioactivity in the 24-hour

urine was $55 \pm 7\%$ of the total radioactivity administered.

HPLC analysis of metabolites. Urine was analyzed for IQ metabolites by HPLC. Metabolites

were assessed using a Beckman HPLC with System Gold software and a 5 μm, 4.6 x 150 mm C-18

ultrasphere column attached to a guard column. Analysis and purification of metabolites required

the use of the following solvent systems at a flow rate of 1 ml/min: solvent system 1, the mobile

phase contained 20 mM ammonium acetate (pH 5.0) in 2% acetonitrile, 0-5 min; 2-5% acetonitrile,

5-23 min; 5-10% acetonitrile, 24-29 min; 10-40% acetonitrile, 35-40 min; 40-2% acetonitrile, 40-45

min; solvent system 2, the mobile phase contained 20 mM ammonium formate (pH 3.1) in 0%

acetonitrile, 0-2 min; 0-4% acetonitrile, 2-22 min; 4-40% acetonitrile, 22-27 min; 40-0%

acetonitrile, 27-32 min; solvent system 3, the mobile phase was the same as solvent system 1,

except the aqueous phase contained 20 mM ammonium formate (pH 3.1); solvent system 4, the

mobile phase contained 20 mM ammonium acetate (pH 5.0) in 5% methanol, 0-3 min; 5-10%, 3-13

min; 10-90%, 18-23 min; 90-5%, 25-30 min; solvent system 5, the mobile phase contained 20 mM

ammonium acetate (pH 5.0) in 10% methanol, 0-15 min; 10-20%, 15-20 min; 20-90%, 20-25 min;

90-10%, 30-35 min; solvent system 6, the mobile phase contained 20 mM ammonium acetate (pH

5.0) in 10% methanol, 0-2 min; 10-20%, 2-7 min; 20-30%, 7-17 min; 30-90%, 20-25 min; 90-10%,

25-30 min; solvent system 7, the mobile phase contained 20 mM ammonium acetate (pH 5.0) in

6% acetonitrile with 6-12% acetonitrile, 0-6 min; 12-35% acetonitrile, 14-19 min; 35-6%

acetonitrile, 20-25 min; solvent system 8, the mobile phase contained 20 mM ammonium formate

(pH 3.1) in 6% acetonitrile with 6-7% acetonitrile, 0-10 min; 7-35% acetonitrile, 10-15 min; 35-6%

acetonitrile, 15-20 min. Radioactivity in HPLC eluents was continuously measured using a FLO-

ONE (PerkinElmer LAS, Inc., Shelton, CT) radioactive flow detector. Data are expressed as a

percent of total radioactivity or nmol recovered after HPLC.

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Processing urine and purification of metabolites. The 24-hour urine samples were treated with an equal volume of methanol and spun at 1,500 xg for 20 min to precipitate protein and debris. After evaporation of methanol in the supernatant, the urine was applied to a C18 solid-phase extraction column (1 gm, PrepSep), previously prepared with methanol/water, washed twice with 6 ml water, and eluted with 6 ml of 100% methanol. The latter was concentrated by evaporation and dissolved in a small volume of methanol. Urine from all animals was compared and analyzed using solvent system 1 (Figure 2, Table 1). Rat metabolites were purified as previously described (Armbrecht et al., 2007).

For purification of mouse urine, metabolites were first separated using solvent system 1, individual fractions collected, samples concentrated, and applied to the next solvent system for further purification. N-Glucuronide was further purified with solvent systems 2 and 4; 5-O-glucuronide with systems 2 and 4, sulfamate with systems 2 and 5; 5-sulfate with systems 3 and 5; Demethyl-IQ and IQ with systems 3 and 6.

Qualitative identification of urinary and liver slice metabolites. Metabolites were identified by their HPLC elution time relative to authentic products previously identified by us and susceptibility to specific treatments (Armbrecht et al., 2007). Urine metabolites were purified before assessing their susceptibility. Slice media was partially purified by extraction with ethyl acetate and metabolites in the aqueous fraction collected from a C18 Sep Pak column before assessing susceptibility. The 5-O-glucuronide was susceptible to *E. coli* beta-glucuronidase (Type VII-A, Sigma, St. Louis, MO) with 10 units enzyme, 50 mM sodium acetate buffer at pH 6.8, and 0.3 ug substrate per 0.1 ml at 37 ^{O}C for 4 hours. 5-Sulfate was susceptible to Abalone sulfatase (Type VIII, Sigma) with 200 units, 50 mM sodium acetate buffer at pH 5.0, and 0.3 ug substrate per 0.1 ml at 37 ^{O}C for 72 hours. N^2-Glucuronide and sulfamate were hydrolyzed with 1 N HCl at 60 ^{O}C for 2 hr.

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To determine whether N-acetyl-IQ was present in mouse urine samples, we synthesized this compound (see below) and then determined its elution profile with different HPLC solvent systems. Although it co-eluted with IQ in our standard solvent system 1, N-acetyl-IQ was separated from IQ in system 3. Under this condition, a peak, which co-elutes with N-acetyl-IQ (about 1% of recovered radioactivity), was observed in mouse urine. Urine samples were treated with carboxyesterase, which converts N-acetyl-IQ to IQ. When urine was incubated with esterase from porcine liver (25 units) in sodium phosphate buffer pH 8.0 for 7 hour at 37°C, no loss of the peak, which co-elutes with N-acetyl-IQ, or increase in IQ was detected. Under these conditions, synthetic N-acetyl-IQ (0.025 mM) and N-acetylbenzidine (0.03 mM) were completely hydrolyzed to their corresponding free amine. Furthermore, while adding synthetic N-acetyl-IQ to urine resulted in complete hydrolysis of synthetic standard to IQ, residual radioactivity (about 1%) previously present remained unhydrolyzed. These results suggest that N-acetyl-IQ is not present in mouse urine. Similar studies with rat urine were unsuccessful. Subsequent studies determined that N-acetyl-IQ was not synthesized from IQ by mouse liver cytosol fractions containing N-acetyl-transferase activity for benzidine (see below).

Liver slice IQ incubation. Mouse liver slices were prepared with a Stadie-Riggs microtome as previously described (Lakshmi et al., 1995b) and incubated with Dulbecco's Modified Eagle Media with high glucose containing 3.7 ug/ml $NaHCO_3$ and 6 ug/ml Hepes. Approximately 150 to 200 mg of liver were placed in a 20 ml scintillation vial with 1 ml of media, gassed with 5% O_2/ 95% CO_2 for 1 minute, and incubated at 37 ^{O}C for 60 min. Media contained 0.06 mM IQ and where indicated 0.5 mM ellipticine or SKF-525A. To stop incubations, 1 ml of cold methanol was added and samples frozen. Thawed samples were sonicated for 15 sec three times, samples spun to remove protein and cell debris, and the supernatant analyzed by HPLC using solvent system 1. Activity is expressed as % of radioactivity recovered by HPLC.

Microsomal IQ incubation. To identify specific pathways for IQ metabolism, liver microsomes were prepared using a previously described procedure (Zenser et al., 1978). Mice were sacrificed by cervical dislocation and open thoracotomy. Samples were stored in small aliquots at –70°C for the assays; unused material was discarded after one freeze-thaw cycle. Microsomes (1 mg/ml) were incubated in sodium phosphate buffer pH 7.4, containing 0.1 mM DETAPAC, 1 mM NADPH, 5 mM $MgCl_2$, and 0.06 mM ^{14}C-IQ for 30 min at 37°C. Microsomes were preincubated with furafylline for 10 min before ^{14}C-IQ addition. The reaction was linear with respect to protein concentration and time and was stopped by addition of an equal volume of methanol with 1 mM vitamin C. After 30 min on ice, the mixture was centrifuged to remove the precipitated protein. Supernatants were analyzed by HPLC, using solvent system 7. Activity is expressed as nmoles or % of radioactivity recovered by HPLC as product. 7-Ethoxyresorufin O-deethylation (EROD) was determined by measuring the fluorescence of resorufin with 0.002 mM ethoxyresorufin (Pohl and Fouts, 1980).

Synthesis of N-acetyl-2-amino-3-methylimidazo[4,5-*f*]quinoline and N-Acetyl-2-amino-imidazo[4,5-*f*]quinoline. To prepare synthetic N-acetyl-IQ, 26 ug ^{14}C-IQ was added to 1 mM acetic anhydride in 0.1 ml pyridine for 15 min at 60°C. The reaction was stopped by the addition of distilled water (0.2 ml). This mixture was later diluted with 10 ml of water, applied to a C18 Sep Pak column, washed with 10 ml of water, and the N-acetylated product was eluted with 3 ml of methanol. The product was purified by solvent systems 8, 7, and 6 and then identified by ESI MS in the positive-ion mode. The product exhibited $[M + H]^+$, $[M + Na]^+$, and $[M + K]^+$ ions at *m/z* 241, 263, and 279, respectively. The $[M + H]^+$ at *m/z* 241 gave rise to prominent ions at *m/z* 199, 184, and 157, representing IQ and consecutive losses of CH_3 and HCN. This fragmentation pattern is consistent with the product being N-acetyl-2-amino-3-methylimidazo[4,5-*f*]quinoline.

N-Acetyl-2-amino-imidazo[4,5-*f*]quinoline was synthesized from Demethyl-IQ as described above for N-acetyl-IQ, except for being only partially purified by immediate methylene chloride extraction due to its lability in methanol and aqueous solutions. The N-acetylated product was identified by ESI MS in the positive-ion mode. The product exhibited an $[M + H]^+$ ion at m/z 227, which gave rise to prominent ions at m/z 185, 158, and 143 representing Demethyl-IQ and consecutive losses of HCN and NH_2-CN. N-Acetyl-2-amino-imidazo[4,5-*f*]quinoline was not stable in aqueous solution and hydrolyzed to Demethyl-IQ.

We also attempted to prepare synthetic N-acetyl-IQ from mouse liver supernatant. The supernatant was saved from the microsomal preparation described above and used to assess N-acetyltransferase activity. Samples were stored in small aliquots at –70°C for the assays; unused material was discarded after one freeze-thaw cycle. Supernatant (1 mg/ml) was incubated (total volume 0.1 ml) in sodium phosphate buffer pH 7.4, containing 1 mM acetyl CoA, 1 mM dithiothreitol, and 0.06 mM ^{14}C-IQ or ^{3}H-N-acetylbenzidine for 60 min at 37°C. The reaction was stopped by addition of 0.1 ml methanol. Supernatants were analyzed by HPLC, using solvent system 8. Under these conditions, 70% of N-acetylbenzidine is converted to N,N$^/$-diacetylbenzidine. However, no formation of N-acetyl-IQ could be detected.

Mass spectral analysis. Metabolites were identified by electrospray ionization (ESI) mass spectrometry (MS). Analyses were performed with a Finnigan TSQ-7000 triple stage quadrupole mass spectrometer (San Jose, CA) equipped with a Finnigan ESI source and controlled by Finnigan ICIS software operated on a DEC alpha workstation. Samples were loop injected onto the ESI source with a Harvard syringe pump, which is continuously infused with methanol at a flow rate of 5uL/min. The skimmer was at ground potential, and the electrospray needle was at 4.5 kV. The heated capillary temperature was 250^{0}C. To obtain collisionally activated dissociation (CAD) tandem mass spectra, the collision energy was set at 22 eV, and argon (2.3 mTorr) was used as

target gas. The product ion spectra were acquired in the profile mode at the scan rate of one scan

per 3 sec.

Statistical Analysis. Data are expressed as a mean $\pm$ S.E. and significant differences evaluated

using a Student's unpaired *t* test with $p < 0.05$.

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Results

HPLC analysis of IQ metabolites in mouse and rat urine. To determine if metabolism of IQ was different in mouse compared to rat, 24-hour urines from each species were examined after dosing with IQ. In Figure 2, top panel, the HPLC elution profile of rat metabolites of IQ previously identified by us and others is illustrated (Luks et al., 1989;Inamasu et al., 1989;Turesky et al., 1986;Armbrecht et al., 2007). These include N-glucuronide, 5-O-glucuronide, sulfamate, and 5-sulfate. While these same metabolites were also present in mouse urine, there was an additional metabolite observed, Demethyl-IQ (Figure 2, bottom panel). Table 1 reports the relative abundance of excreted metabolites using HPLC data illustrated in Figure 2. The relative abundance of metabolites in these species was different. For rat, the proportion of metabolites excreted was 5-O-glucuronide > sulfamate > 5-sulfate > N-glucuronide. The presence of Demethyl-IQ in rat urine was not confirmed by mass spectrometry. For mouse, the proportion of metabolites excreted was 5-O-glucuronide > Demethyl-IQ > sulfamate > N-glucuronide > 5-sulfate. Using HPLC solvent system 3, the amount of unmetabolized IQ in mouse urine was 2.3 ± 0.4 % of recovered radioactivity. N-Acetyl-2-amino-3-methylimidazo[4,5-*f*]quinoline was not detected. The urinary IQ metabolite profile in mouse and rat is different and Demethyl-IQ is a major new metabolite in mouse.

To determine if the route of IQ administration is responsible for the lack of Demethyl-IQ formation in rat, rats were administered IQ by ip injection. In these experiments, the proportion of metabolites excreted was sulfamate ($48 \pm 1\%$) > 5-O-glucuronide ($15 \pm 0.5\%$) = 5-sulfate ($15 \pm 0.5\%$) > N-glucuronide ($4.8 \pm 0.4\%$). The amount of radioactivity that co-eluted with Demethyl-IQ was $1.9 \pm 0.2\%$ of the total radioactivity after HPLC and not further identified.

Identification of urinary IQ metabolites. Rat metabolites were identified by their HPLC elution profile relative to authentic products previously identified by us (Armbrecht et al., 2007) and their

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susceptibility to specific treatments. The N-glucuronide and sulfamate were susceptible to

treatment with 1 N HCl at 60 ^{O}C, while 5-O-glucuronide and 5-sulfate were susceptible to beta-

glucuronidase and sulfatase, respectively. Rat metabolites were only susceptible to the treatments

indicated.

Mouse metabolites were further identified by their elution profile relative to rat metabolite

standards (Armbrecht et al., 2007), susceptibility to specific treatments described above for rat, and

ESI MS spectra in Figure 3. In the negative-ion mode, the 12 min peak exhibited an [M - H] $^-$ at m/z

373 and gave prominent ions at m/z 239 and 197, representing N-acetyl-IQ and IQ. The 12 min

metabolite is IQ-N^2-glucuronide (Figure 3, panel A). The 17 min peak, in the negative-ion mode,

yielded deprotonated [M – H]$^-$ molecular ion at m/z 389 and gave prominent ions at m/z 213 and

198, representing 5-OH-IQ and loss of CH_3. This is consistent with the 17 min product being IQ-5-

O-glucuronide (Figure 3, panel B). The 19.5 min peak, in the negative-ion mode, exhibited an [M –

H]$^-$ ion at m/z 277 and gave rise to prominent ions at m/z 197 and 182, representing IQ and loss of

CH_3. The ion at m/z 80 corresponded to SO_3. This fragmentation pattern is consistent with the 19.5

min peak being IQ-sulfamate (Figure 3, panel C). For the 26 min peak, the negative-ion mode

displayed an [M – H]$^-$ ion at m/z 293 and gave prominent ions at m/z 213 and 198, representing 5-

OH-IQ and loss of CH_3. The ion at m/z 80 corresponds to SO_3. In the positive-ion mode, the

product yielded [M + Na]$^+$ at m/z 317. The 26 min metabolite is IQ-5-sulfate (Figure 3, panel D).

Urinary IQ was identified by its co-elution with authentic commercial material and by its mass

spectra (Figure 3, panel E). In the positive-ion mode, the [M + H]$^+$ at m/z 199 gave prominent ions

at m/z 184, 157, and 131 representing consecutive losses of CH_3, HCN, and C_2H_2, respectively. The

spectra of the commercial starting material were identical to that of urinary IQ. The ESI MS

fragmentation patterns of all the metabolites matched their corresponding metabolite in rat. This is

consistent with the mouse and rat metabolites being identical to one another and not isomers.

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The ESI MS spectra of the new metabolite eluting at 31.5 min is illustrated in Figure 4. In the positive mode, this urinary metabolite exhibited [M + H]$^+$ at *m/z* 185. The product ion spectra of *m/z* 185 along with its fragmentation pattern is consistent with it being 2-amino-imidazo[4,5-*f*]quinoline (Demethyl-IQ).

Analysis of IQ metabolites produced by mouse liver slices. To further evaluate IQ metabolism in the mouse, liver slices were assessed (Figure 5). The HPLC elution profile indicates the following metabolites were present: 5-O-glucuronide, sulfamate, and Demethyl-IQ. 5-O-Glucuronide and sulfamate were further identified by their susceptibility to beta-glucuronidase and acid treatment, respectively. Table 2 reports the % of total radioactivity recovered after HPLC for each metabolite. P450 inhibitors, 0.5 mM ellipticine or SKF-525A, were incubated with slices to determine the role of these enzymes in IQ metabolism. 5-O-Glucuronide synthesis was significantly inhibited by ellipticine (19 % of Control radioactivity). SKF-525A did not prevent glucuronide formation. Sulfamate synthesis was not altered by either inhibitor, but Demethyl-IQ production was significantly decreased by ellipticine (44% of Control radioactivity) and SKF-525A (56%). These results demonstrate mouse liver slice synthesis of three IQ metabolites with 5-O-glucuronide and Demethyl-IQ being produced by P450.

Evaluation of Demethyl-IQ formation by mouse liver microsomes. To examine P450 N-demethylase catalyzed metabolism of IQ in more detail, liver microsomal metabolism was examined. Consistent with liver slice data, Demethyl-IQ was a product of liver microsomal metabolism and its formation was inhibited by ellipticine (Figure 6). Demethyl-IQ was the only metabolite of IQ observed. In similar studies with rat microsomes, Demethyl-IQ was not detected (not shown). ESI MS spectra of the microsomal product was identical to that illustrated in Figure 4. To determine the P450(s) responsible for Demethyl-IQ formation, inhibitors of different P450s previously used to assess N-demethylation were examined (Iribarne et al., 1996;Yamagata et al.,

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1998). In Table 3, ellipticine, a P450 1A inhibitor, was the only inhibitor to completely prevent Demethyl-IQ formation at 0.02 mM. At this concentration, inhibition was also observed with alpha-naphthoflavone (27 % of Total nmoles; 1A1/1A2 inhibitor), SKF-525A (74%; nonspecific inhibitor), and ketoconazole (64%; 3A inhibitor). No inhibition was detected with 0.02 mM furafylline (1A2 inhibitor) or 0.1 mM sulfaphenazole (2C inhibitor). To assess the efficiency of ellipticine inhibition of N-demethylation, a range of concentrations was used and a Dixon plot determined the Ki to be 0.001 mM. EROD is thought to represent 1A1 activity and was used to further evaluate ellipticine inhibition. Kinetic plots determined EROD Ki values for ellipticine and furafylline to be 0.0008 and 0.011 mM, respectively. These results are consistent with P450 1A1 mediating N-demethylation of IQ in mouse liver.

Analysis of mouse urine for Demethyl-IQ metabolites. To determine whether Demethyl-IQ is further metabolized, purified Demethyl-IQ (10 ug) was injected i.p. into a mouse and a 24-hour urine collected. No metabolism of Demethyl-IQ was detected (not shown). We have synthesized N-acetyl-Demethyl-IQ and determined that it elutes at 41.5 min (see Figure 2). Thus, this N-acetylated product was not detected in urine from a mouse injected with either IQ or Demethyl-IQ. Additional studies demonstrated that Demethyl-IQ was not metabolized by liver microsomes or N-acetylated by liver cytosol (not shown). Thus, Demethyl-IQ does not further contribute to mouse metabolites derived from IQ.

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Discussion

This study examined mouse IQ metabolism and discovered a previously unidentified rodent metabolite. All of the major urinary metabolites previously identified in rats were also found in mice. Although the 5-O-glucuronide represented a similar percentage of total radioactivity excreted in both species, the rat excretion of sulfamate and 5-sulfate exceed that in mouse by 3- and 4-fold, respectively. The major difference between these species was the large amount of Demethyl-IQ synthesized by mouse (26 % of the total radioactivity excreted), but not rat. In mouse, excretion of Demethyl-IQ exceed that of sulfamate by more than 2-fold. The presence of Demethyl-IQ in urine was consistent with its synthesis by mouse liver slices and microsomes. No further metabolism of Demethyl-IQ could be demonstrated by an intact mouse or liver microsomes. However, incubations demonstrating mutagenicity of Demethyl-IQ were supplemented with the S9 fraction of liver from Aroclor 1254-treated rats, suggesting that metabolic activation of Demethyl-IQ occurs (Barnes et al., 1985). Demethyl-IQ is a major metabolite in monkey (Snyderwine et al., 1992). Mice have a high capacity for metabolizing IQ since little IQ was found unmetabolized (1.2 ± 0.3 %) in urine following administration of a high dose of IQ (40 mg/kg). Results are consistent with mouse IQ metabolism being significantly different from rat and with Demethyl-IQ being a major new rodent metabolite unique to the mouse.

It is not clear if rats N-demethylate IQ. While we reported Demethyl-IQ in rat urine (Table 1), this was not confirmed by ESI MS. In a prior study in which rats were given an oral dose of IQ (40 mg/kg), the 24-hour urine was thought to contain Demethyl-IQ along with some other non-polar metabolites, including IQ, N-acetyl-IQ, and 7-OH-IQ (Inamasu et al., 1989). This non-polar urine fraction represented less than 1% of the dose administered. The metabolites were only identified by their co-elution with synthetic standards. Another study by this group provided MS and NMR structural identification for the sulfamate, 5-O-glucuronide, 5-sulfate, and N-glucuronide conjugates

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of IQ, but no further mention of Demethyl-IQ or the other non-polar metabolites (Luks et al., 1989).

Studies of rat IQ metabolism by other investigators have not demonstrated the formation of

Demethyl-IQ (Alexander et al., 1989). However, rat liver microsomes N-demethylate dacarbazine

by 1A enzymes (Yamagata et al., 1998). Thus, structural proof for the presence of Demethyl-IQ in

rat urine has not been demonstrated and if present, N-demethylation would only be a minor pathway

compared to mouse.

Results suggest that the route of IQ administration does not effect the formation of

Demethyl-IQ in the rat. Using either oral or ip administration of IQ, less than 2% of radioactivity

recovered after HPLC co-eluted with Demethyl-IQ compared to $26 \pm 1.6\%$ in mouse. Because this

could not be confirmed by ESI MS, it is not clear if rats N-demethylate IQ. While excretion of

sulfamate and 5-O-glucuronide was similar after oral IQ administration in the rat, the excretion of

sulfamate was 3-fold greater than 5-O-glucuronide following ip injection. This change in

metabolite distribution is likely due to higher plasma concentrations of IQ following ip injection

with higher concentrations of IQ within hepatocytes saturating P450 metabolism and allowing more

sulfamate formation.

Studies with monkey identified three Demethyl-IQ metabolites in urine (Snyderwine et al.,

1992). They were Demethyl-IQ, Demethyl-IQ N-glucuronide, and Demethyl-7-oxo-IQ. The latter

was thought to be derived from intestinal bacterial metabolism of Demethyl-IQ. Approximately, 16

to 22% of the dose given at 2.2 umol/kg IQ in two monkeys was derived from N-demethylation. In

contrast to monkey, we observed no further metabolism of Demethyl-IQ. Human hepatocytes N-

demethylate a HCA structurally similar to IQ (Langouet et al., 2001). Demethyl-IQ is mutagenic

(Barnes et al., 1985). Thus, human, monkey and mouse N-demethylate HCAs.

While N-acetylation is a characteristic feature of aromatic amine metabolism, this pathway

does not appear to be active in heterocyclic amine metabolism. We were unable to detect N-acetyl-

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IQ in mouse urine or as a product of mouse liver cytosol N-acetyltransferase activity. The lack of

NAT1 and NAT2 N-acetylation of IQ and MeIQx has been attributed to their 3-methyl group.

Results with N-acetylation of *ortho*-toludine compared to *para*-toludine suggested that there is

steric hindrance of the exocyclic amine group resulting from methyl group substitution ortho to the

amine (Hein et al., 1993). However, since N-acetylation of Demethyl-IQ was not demonstrated

either in vitro or in vivo, steric hindrance by the 3-methyl group does not appear to be the reason for

the lack of N-acetylation of these heterocyclic amines. In aqueous solutions, chemically

synthesized N-acetyl-IQ was stable, while N-acetyl-Demethyl-IQ was not stable. In addition, the

N-glucuronides of aromatic amines are quite acid labile, i.e., the N-glucuronide of N-

acetylbenzidine has a half-life at pH 5.5 of about 8 min (Babu et al., 1995), while N-glucuronides of

these heterocyclic amines are relatively acid stable (see methods). Results suggest that the

chemistry involving the exocyclic amine of these heterocyclic compounds is different from

aromatic amines and may also contribute to the lack or low rate of N-acetylation of heterocyclic

amines. For example, the electron withdrawing effects of the nitrogen substituents of the imidazole

ring may reduce the reactivity of the exocyclic amine or the amine may preferentially exist as an

imine tautomer, making it less likely to be N-acetylated.

A variety of inhibitors were used to determine the P450(s) responsible for N-demethylation

of IQ. The 1A inhibitor ellipticine was effective in liver slices and also in microsomal incubations

with the Ki for Demethyl-IQ formation 0.001 mM. At the concentration of 0.02 mM used for all

inhibitors (Table 3), other compounds exhibited inhibition with alpha-naphthoflavone >

ketoconazole > SKF-525A. The former, like ellipticine, is an inhibitor of both 1A1 and 1A2 and

would be expected to be an effective inhibitor of 1A1 catalyzed reactions. Ketoconazole has been

reported to be an effective inhibitor of 1A2 in rat (Kobayashi et al., 2003) and may exhibit some

1A1-like activity in mouse. SKF-525A is a nonspecific inhibitor. The lack of effect of furafylline,

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a 1A2 selective inhibitor, further demonstrates the apparent selectivity of 1A1 for IQ N-demethylation. Although P450 3A does not mediate mouse liver IQ N-demethylation, previous studies have shown 3A to be expressed in this tissue and to catalyze N-demethylation of cocaine (Martignoni et al., 2006;Pellinen et al., 1994). EROD activity demonstrated preferential inhibition by ellipticine compared to furafylline similar to that observed for IQ N-demethylation. In addition, mouse liver benzo[alpha]pyrene hydroxylase, an activity attributed to 1A1, was inhibited by 0.005 mM alpha-naphthoflavone, but not 0.02 mM furafylline (Tsyrlov et al., 1993). That study also demonstrated preferential inhibition of mouse 1A2 by furafylline compared to 1A1. Thus, mouse IQ N-demethylation appears to be catalyzed by hepatic P450 1A1.

Because of our interest in the role of colitis in colon cancer (Clapper et al., 2006;Cooper et al., 2000), we assessed IQ metabolism in female C57Bl/6 mice administered DSS in their drinking water. After three cycles of DSS to induce colitis, an oral dose of 40 mg/kg IQ was administered and analysis conducted as described in this report. While urinary 5-O-glucuronide excretion decreased from 30.8 ± 2.5 % in control to 17.2 ± 2.1 % of total radioactivity for DSS-treated mice, the distribution of other excreted metabolites was not different. These results are consistent with a similar study in which rats were administered DSS at 3 % in drinking water for 7 days. Decreases in several hepatic P450 enzyme activities, including P450 1A1 and A2 phenacetin O-deethylation activity, were reported (Masubuchi and Horie, 2004). Inflammation decreases P450-mediated metabolism (for review see (Morgan, 2003) and this is a likely mechanism by which DSS decreased 5-O-glucuronide formation.

In conclusion, this study demonstrated for the first time that N-demethylation is an important pathway for IQ metabolism in mouse, possibly mediated by P450 1A1. However, the constitutive expression of 1A1 in mouse liver is low (Dey et al., 1999) and additional studies may be needed to determine the P450 responsible for Demethyl-IQ formation. Demethyl-IQ is

mutagenic (Barnes et al., 1985). N-Demethylation is a prominent pathway in mouse, monkey

(Snyderwine et al., 1992), and human (Langouet et al., 2001) and may play a role in IQ

tumorigenicity in these species. Differences in metabolism between mouse and rat, especially

Demethyl-IQ formation, suggests more similarity of mouse to human metabolism of HCAs than rat

and that the mouse is a useful model for testing tumorigenicity of these chemicals.

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Footnotes

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Legends for Figure

Figure 1. Structures of IQ and its metabolites.

Figure 2. Urinary excretion of ^{14}C-IQ in rat and mouse. Mice and rats were administered an oral dose of 40 mg/kg ^{14}C-IQ and a 24-hour urine collected. A representative HPLC profile from each treatment group is shown using solvent system 1. The peaks were tentatively identified by retention times and confirmed by mass spectrometry and susceptibility to specific treatments.

Figure 3. ESI MS spectra of IQ urinary metabolites. Panels A to E represent purified urinary metabolite IQ-N^2-glucuronide, IQ-5-O-glucuronide, IQ-sulfamate, IQ-5-sulfate, and IQ, respectively. All spectra were obtained in the negative-ion mode, except IQ in panel E is in the positive-ion mode.

Figure 4. Positive ion ESI MS of Demethyl-IQ purified from mouse urine. The product ion spectra of m/z 185 is illustrated along with its fragmentation pattern. Results confirm that Demethyl-IQ is 2-amino-imidazo[4,5-f]quinoline.

Figure 5. Mouse liver slice metabolism of IQ. Slices were incubated with 0.06 mM ^{14}C-IQ for 60 min at 37°C in the presence and absence of 0.5 mM ellipticine. A representative HPLC profile from each treatment group is shown using solvent system 1. Peaks were identified by retention times and susceptibility to specific treatments.

Figure 6. Mouse liver microsomal metabolism of IQ. Microsomes were incubated with 0.06 mM ^{14}C-IQ for 30 min at 37°C in the presence and absence of 0.01 mM ellipticine. A representative HPLC profile from each treatment group is shown using solvent system 7. The Demethyl-IQ peak were tentatively identified by its retention time and confirmed by mass spectrometry.

Table 1

IQ Metabolites in rat and mouse urine

Urine from rats and mice were analyzed by HPLC as illustrated in Figure 2. The relative abundance of excreted metabolites is provide by table entries as the mean $\pm$ SE of 4 rats and 4 mice. Rodents were administered ^{14}C-IQ and placed in metabolic cages for a 24-hr urine collection.

Condition	N-Glucuronide	5-O-Glucuronide	Sulfamate	5-Sulfate	Demethyl-IQ
		% of Total Urine Radioactivity			
Rat	5.0 ± 0.6	35 ± 1	$30 \pm 2^*$	$15 \pm 0.9^*$	$0.9 \pm 0.2^*$
Mouse	5.2 ± 0.4	36 ± 1	11 ± 1	3.8 ± 0.1	26 ± 2

[*]Significantly different than mouse ($P < 0.05$).

Table 2

IQ metabolites produced by mouse liver slices

Slices were incubated for 60 min at 37°C with 0.06 mM IQ and where indicated 0.5 mM

ellipticine or SKF-525A. Samples were analyzed by HPLC and data recorded as % of total

radioactivity recovered. Entries represent mean $\pm$ SE of determinations from 3 mice.

Condition	5-O-Glucuronide	Sulfamate	Demethyl-IQ
	% of Total Radioactivity		
Control	4.3 $\pm$ 0.2	5.7 $\pm$ 0.3	5.0 $\pm$ 0.6
+ Ellipticine	0.8 $\pm$ 0.2[*]	4.2 $\pm$ 0.9	2.2 $\pm$ 0.4[*]
+ SKF-525A	2.9 $\pm$ 0.5	5.5 $\pm$ 0.5	2.8 $\pm$ 0.4[*]

[*]Significantly different from Control (P < 0.05).

Table 3

P450 Inhibition of Mouse Microsomal Demethyl-IQ Formation

Incubation was conducted for 30 min at 37°C with 0.06 mM IQ. Furafylline was

preincubated for 10 min before addition of IQ. For those incubations, the microsomal activity was

0.80 $\pm$ 0.06 nmoles, which is not different from the control value below. Samples were analyzed by

HPLC and data are expressed as mean nmoles Demethyl-IQ $\pm$ SE with n = 3 to 5.

Test Agents	Type of P450 inhibitor	Concentraion uM	Activity nmoles	% of Total nmoles
Control			0.74 $\pm$ 0.04	100
Ellipticine	1A	10	0.02 $\pm$ 0.01[*]	3
		20	ND[c]	0
Furafylline	1A2	20	0.70 $\pm$ 0.04	95
Alpha-Naphthoflavone	1A	20	0.20 $\pm$ 0.02[*]	27
SKF-525A	Nonspecific	20	0.55 $\pm$ 0.06[*]	74
		100	0.21 $\pm$ 0.02[*]	28
Ketoconazole	3A	20	0.47 $\pm$ 0.06[*]	64
		100	0.17 $\pm$ 0.04[*]	23

| Sulfaphenazole | 2C | 20 | 0.65 ± 0.03 | 88 |
| | | 100 | 0.79 ± 0.05 | 107 |

ND, not detected; [*]$p < 0.05$ compared to control

FIGURE 1

Structures of IQ and Its Metabolites
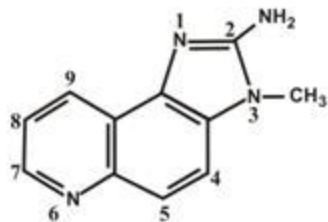
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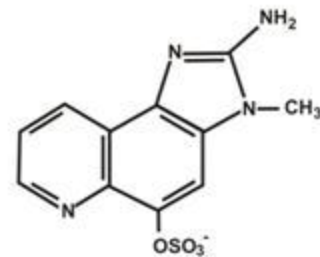
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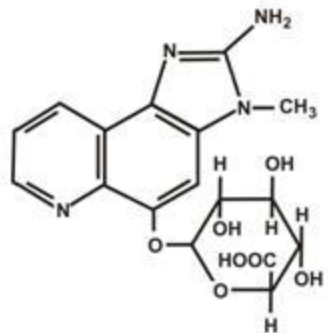
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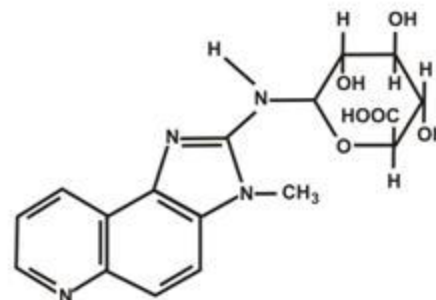
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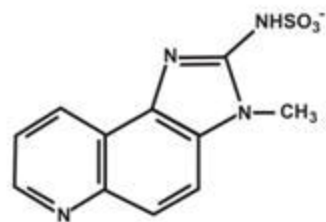
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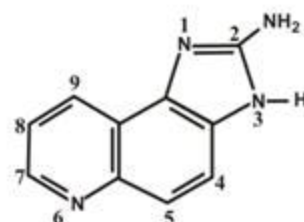
Demethyl-IQ

FIGURE 2
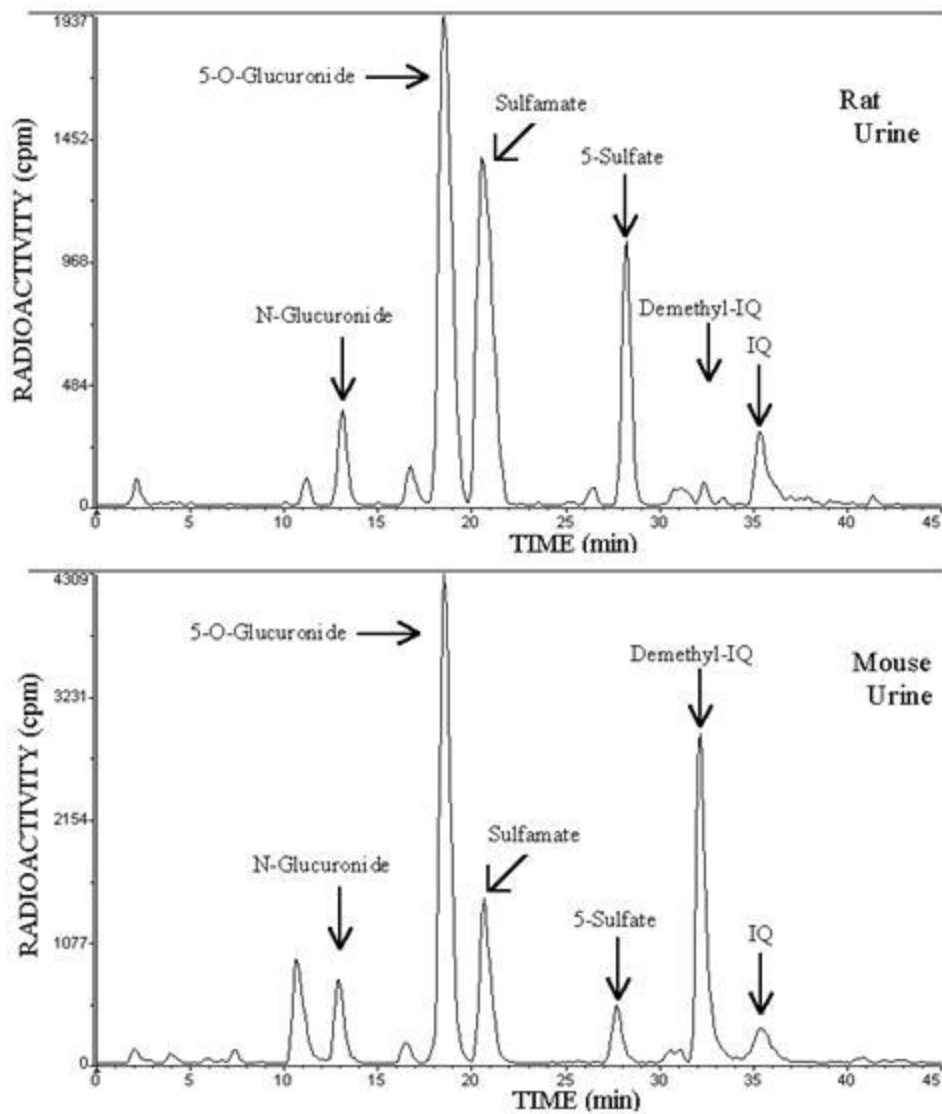
FIGURE 3
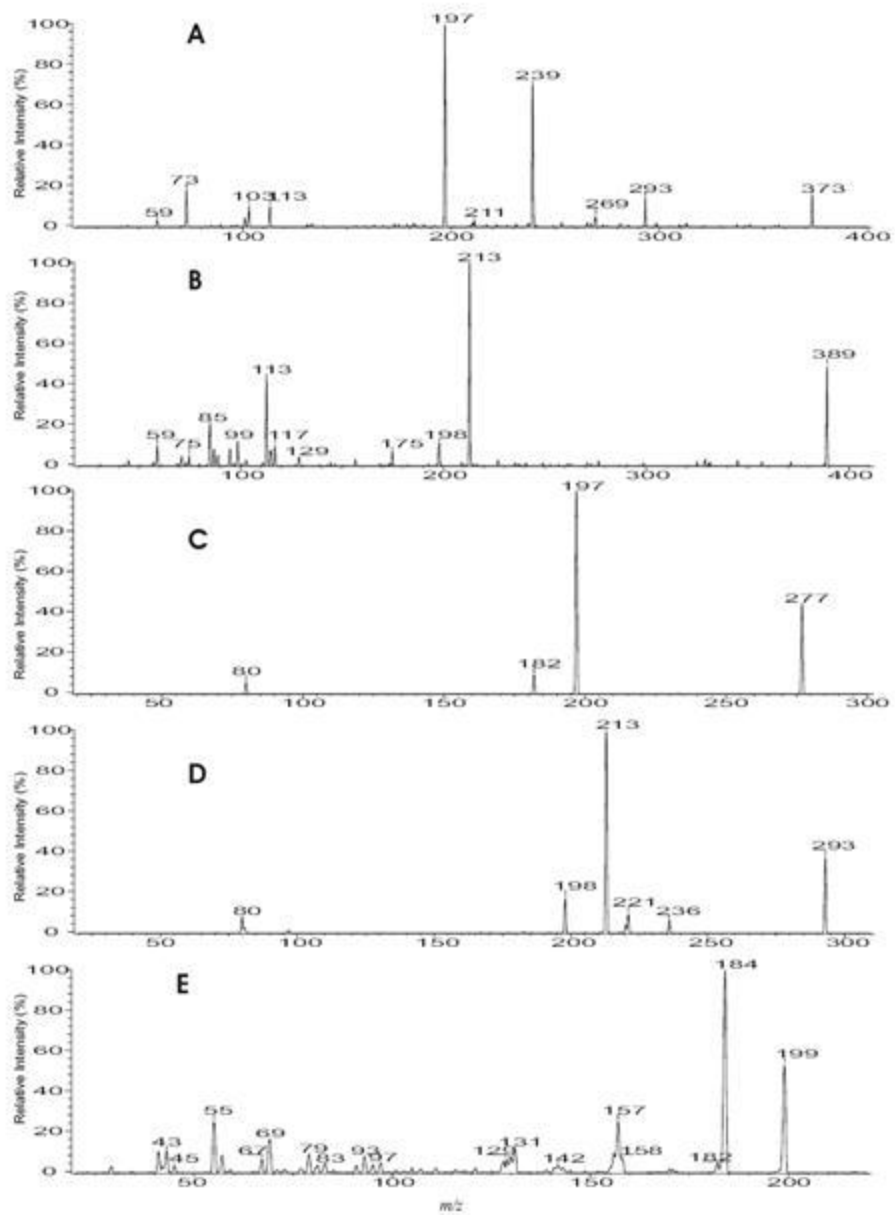
FIGURE 4

FIGURE 5

FIGURE 6