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Metabolism of 4-methylimidazole in Fischer 344 rats and B6C3F1 mice

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ABSTRACT

4-Methylimidazole (4-MeI) is a widely used chemical, also identified as a by-product of heating foods. In cancer bioassays, 4-MeI induced lung tumors in mice, but not in rats. To establish if metabolic differences could explain species difference in carcinogenicity, this study investigated metabolism of 4-MeI in rat and mouse lung and liver microsomes and S-9 fractions, and in vivo in rats and mice. No metabolites were detected in rat or mouse lung and liver microsomes, or lung S-9 fractions. Male and female F-344 rats and B6C3F1 mice were administered 50 and 150 mg/kg [^{14}C] 4-MeI by gavage. Excreta, exhaled CO_2 and volatiles were collected for 48 h. Elimination was mainly via urine, with 79–89% of the radioactivity in urine in rats and 41–70% in mice. Most of the radioactivity (71–88%) in urine was unchanged 4-MeI. Additional radioactive peaks (the largest metabolite was 8–18%) were characterized by LC-MS/MS as 4-hydroxymethylimidazole, its glucuronide, and other oxidized products, including methylhydantoin. 4-MeI was largely excreted unchanged in rats and mice with limited oxidative metabolism and conjugation. 4-MeI was not oxidized in subcellular fractions from rat and mouse lung and liver. Overall, the metabolism of 4-MeI appeared similar between rats and mice.

1. Introduction

4-Methylimidazole (4-MeI) is used in the manufacture of pharmaceuticals, photographic chemicals, dyes and pigments, cleaning and agricultural chemicals, and rubber and can be formed in heated foods such as coffee and in some caramel colors used in foods and beverages (Hengel and Shibamoto, 2013). It is formed by the Maillard reaction of D-glucose and ammonia. While a variety of acute effects have been described in animals administered 4-MeI, including lethality, CNS effects, and seizures, a potential concern is the effect of 4-MeI chronic low dose exposure. The National Toxicology Program investigated the carcinogenicity of 4-MeI in a lifetime bioassay in male and female Fischer 344 rats and B6C3F1 mice with administration in feed for 2 years (Chan et al., 2008; NTP, 2007). There was no evidence of increased neoplasia in male rats. There was equivocal evidence of carcinogenic activity on dietary administration in female Fischer 344 rats based on the incidence of mononuclear cell leukemia at the highest dose of 5000 ppm. Dietary administration caused increased incidences of adenomas/carcinomas in the mouse lung in both male and female B6C3F1 mice.

Genotoxicity evaluations of 4-MeI in *Salmonella typhimurium* strains TA97, TA98, TA100, and TA1535 were negative with and without metabolic activation with rat or hamster metabolic activation systems (NTP, 2004, 2007) No consistent or significant increases in micronuclei in erythrocytes were found in bone marrow from male rats and mice administered 4-MeI by i.p. injection 3 times at 24-h intervals, or in feed for 13 weeks (NTP, 2007). Beevers and Adamson (2016) recently reported absence of mutagenicity in five *Salmonella typhimurium* strains, with and without metabolic activation from rat and mouse liver and lung S9.

In the male Fischer 344 rat, [2–^{14}C] 4-MeI is largely excreted unchanged in urine, with a minor metabolite tentatively identified as the sulfate conjugate (Yuan and Burka, 1995). After gavage administration of 50 mg/kg, the cumulative recovery of radioactivity at 48 h was 85% in urine, 0.6% as CO_2, 0.04% as exhaled volatile organics, 3% in feces and 0.8% remaining in the body. No metabolism studies have been reported in the mouse, or in female rats. The pharmacokinetics of [2–^{14}C] 4-MeI were determined by detection of radioactivity, and indicated that plasma radioactivity was largely [2–^{14}C] 4-MeI. Following a 5 mg/kg i.v. dose, the plasma elimination half-life was 1.8 h. The bioavailability following oral administration was estimated to be 60–70% following doses of 5, 50, and 150 mg/kg. These authors indicated that the metabolism of 4-MeI and renal clearance were most likely saturable processes.

The metabolism of 4-MeI to a potentially reactive epoxide has been proposed based on analogy to other 5-membered heterocyclic compounds by Chan et al. (2008) (Fig. 1). However, this postulated route has not been substantiated by direct investigation of 4-MeI metabolism.

Differences in the susceptibility of the mouse and rat lung during the

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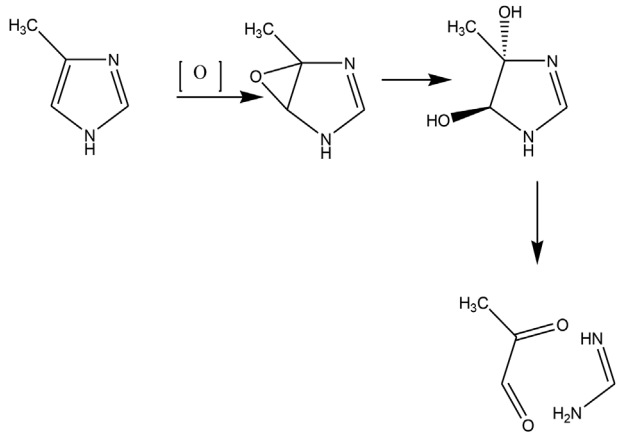

Fig. 1. Postulated metabolism of 4-MeI to an epoxide, and subsequent hydrolysis to give methylglyoxal (from Chan et al., 2008).

course of cancer bioassays have been observed, where differences in the metabolism of a chemical between rats and mice are thought to contribute to the susceptibility. In mice, the exocrine bronchiolar cell or club cell, the primary location of CYP enzymes in the lungs, is present in much greater numbers than in rats, and in turn in human lung, (Plopper et al., 1980a; b; Plopper et al., 1992), resulting in substantially higher metabolism in the mouse lung compared to the rat lung. For example, trichloroethylene undergoes more extensive oxidation in mouse lung than rat lung via CYP2E1 expressed in mouse lung club cells (Green et al., 1997). Methylene chloride undergoes extensive metabolism via glutathione transferase theta in mouse lung compared with rat lung, and this is thought to be a key difference in the response difference between rat and mouse (Green, 1997). CYP enzymes that are known to be substantially different between mouse, rat and human lung are CYP2F1 in human lung, CYP2F2 in mouse lung, and CYP2F4 in rat lung (Carlson, 2008). There are a number of chemicals that display club cell toxicity and increased incidences of lung tumors in mice, including naphthalene, methylene chloride, trichloroethylene and styrene. None of these chemicals show corresponding club cell necrosis or lung tumors in the rat (Carlson, 2008).It has been demonstrated that CYP2F2 in mouse lung club cells is responsible for the metabolism of styrene to toxic metabolites in mouse lung, leading to mouse lung tumors (Cruzan et al., 2002, 2009). In these instances, a comparison of the metabolism in humans vs. rodents becomes essential for risk assessment.

While CYP catalyzed oxidation of 4-MeI could play a role in its metabolism, the inhibition of various CYP activities has been reported in vitro. 4-MeI inhibited p-nitrophenol oxidation characteristic of CYP2E1 in rat liver microsomes with a Ki of 23 μM in a non-competitive manner (Hargreaves et al., 1994). 4-MeI administered to male rats had an inhibitory effect on the clearance of tolbutamide in vivo, and in vitro, 4-MeI inhibited tolbutamide metabolism in human liver microsomes (Back and Tjia, 1985; Back et al., 1988). The objectives of this study were to investigate the metabolism of 4-MeI in male and female rats and mice, and to determine whether there are species differences in pharmacokinetics and metabolism that might explain differences in susceptibility to 4-MeI effects. The in vitro metabolism of 4-MeI was investigated in male and female rat and mouse liver and lung microsomes as well as in liver and lung S-9 fractions. LC-MS/MS methods were developed to evaluate the metabolism of 4-MeI. Methods for trapping a potential metabolite methylglyoxal with o-phenylenediamine, and for trapping metabolites by addition of GSH were explored. Metabolism and disposition of 4-MeI was examined in male and female Fischer 344 rats and B6C3F1 mice administered a single dose of [4–^{14}C] 4-MeI (50 or 150 mg/kg by gavage).

2. Methods

2.1. Chemicals

4-Methylimidazole (99.8%) was obtained from Sigma Aldrich. MC-1001575 [4–^{14}C] 4-MeI Lot 270-133-0099-A-20120523-JIM was obtained from Moravek Biochemicals (Brea, CA). The specific activity was 9.9 mCi/mmol 4-Methylhydantoin and 4-hydroxymethylimidazole were purchased from Sigma Aldrich. 2-Methylhydantoic acid was synthesized by reaction of alanine with potassium cyanate, and was characterized by NMR spectroscopy as described by Nielsen et al. (1993). Propranolol, phenacetin, acetaminophen, verapamil, 7-ethoxycoumarin, 7-hydroxycoumarin, 7-ethoxyresorufin, resorufin and chlorzoxazone were obtained from Sigma Aldrich (St. Louis, MO) and 6-hydroxychlorzoxazone was obtained from BD Gentest.

2.2. Animals

All studies were conducted at RTI International (RTP, NC) and were approved by the Institutional Animal Care and Use Committee. Animals were housed in a facility that is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal procedures were in accordance with the "Guide for the Care and Use of Laboratory Animals' (Institute for Laboratory Animal Research, 2011). Animals were provided Purina 5002 feed and tap water (City of Durham) ad libitum, and were housed in polycarbonate cages with stainless steel bar lids accommodating a water bottle, and with Sani Chips bedding. All animals were acclimated in the RTI animal facility for 7 days prior to use.

2.3. In vitro investigations

2.3.1. Microsomes and S-9

Male F-344 rat and male B6C3F1 mouse liver microsomes used in initial studies were obtained from Xenotech (Lenaxa KS). For later studies, microsomes were prepared in-house. Male and female F-344 rats and B6C3F1 mice were obtained from Charles River (Raleigh NC) at approximately 8 weeks of age. Animals were euthanized by exposure to CO_2 and livers and lungs were removed for preparation of liver and lung microsomes, using a method modified from Matsubara et al. (1974). Mouse lung and liver microsomes from male and female B6C3F1 mice were also prepared. Microsomes were stored frozen at −80 °C.

S-9 fraction from Male Fischer rat liver and lung and S-9 from B6C3F1 mouse liver and lung was provided by Celsis | In Vitro Technologies (Baltimore, MD) (Beevers and Adamson, 2016).

Protein concentration was determined by a modified Bradford (1976) method using a DC Protein Assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as a standard.

For microsomal metabolism assays, incubations were conducted in triplicate, with 1, 10, or 100 μM 4-MeI, microsomes (1 mg protein/mL), and NADPH at 1 mM or an NADPH generating system consisting of 0.5 mL of 100 mM NADP, 0.5 mL of 500 mM Glucose-6-phosphate, 0.5 mL of 100 units/mL Glucose-6-phosphate dehydrogenase, in 100 mM Tris-HCl buffer, pH 7.4. For incubations with glutathione, a final concentration of 1 mM GSH was added. The total incubation volume was 500 μL. Reactions were quenched by addition of acetonitrile (500 μL). For positive control substrates to ensure activity in microsomal fractions, verapamil (1 μM), propranolol (1 μM), chlorzoxazone (75 μM), 7-ethoxycoumarin (500 μM), 7-ethoxyresorufin (10 μM) and phenacetin (50 μM) were also incubated as described above, with detection of metabolism by LC-MS. Verapamil and propranolol metabolism were monitored using MRM transitions *m/z* 455 → 165, and *m/z* 260 → 116, respectively. Phenacetin O-deethylation was monitored by the appearance of acetaminophen (*m/z* 152 → 110), and chlorzoxazone hydroxylase was monitored by determination of hydroxychlorzoxazone

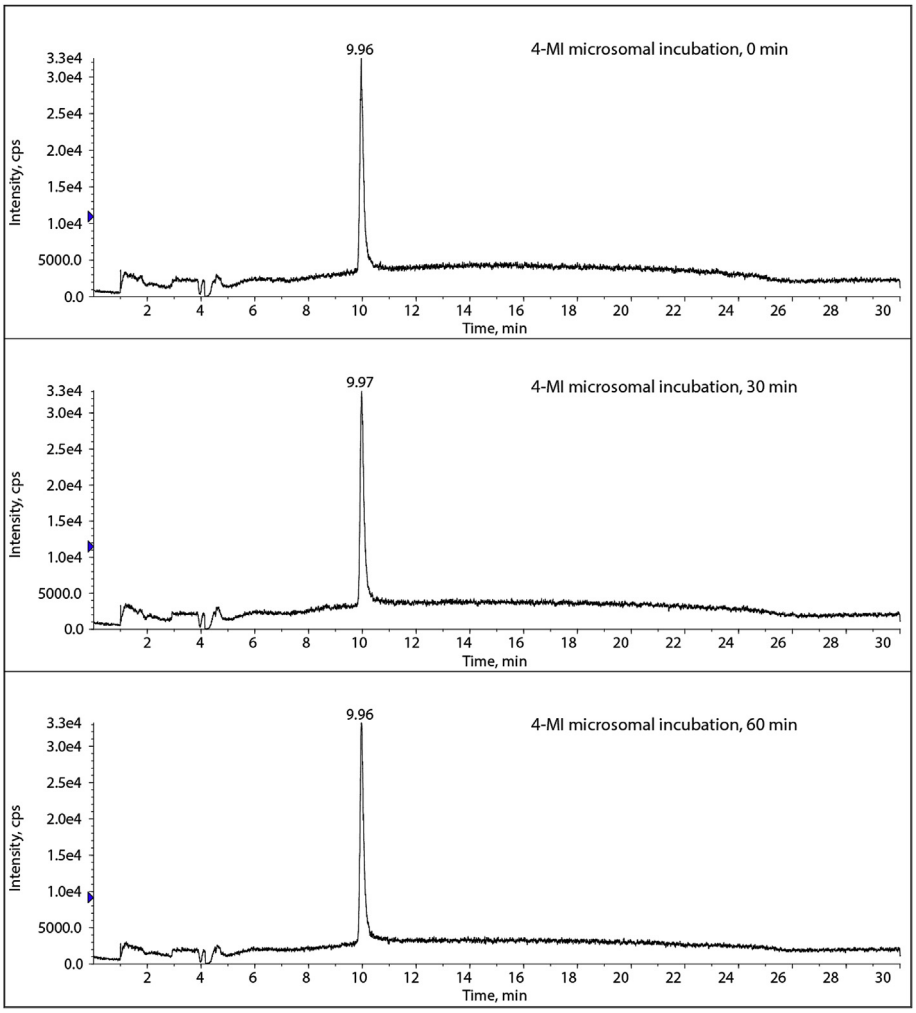

Fig. 2. LC-MS analysis of male mouse liver microsomal clearance of 4-MeI.

Table 1
Ethoxycoumarin O-dealkylation activity in liver and lung microsomes.

Microsome Preparation	Ethoxycoumarin O-dealkylation Activity (pmol/mg protein/min)[a]
Xenotech F344 Male Rat Liver	1470 ± 12.2[b]
F344 Male Rat Liver	3547 ± 122
F344 Female Rat Liver	1433 ± 23.0
B6C3F1 Male Mouse Liver	2764 ± 105
B6C3F1 Female Mouse Liver	2959 ± 82.0

Microsome Preparation	Phenacetin O-dealkylation Activity (pmol/mg protein/min)[a]	Ethoxyresorufin O-dealkylation Activity (pmol/mg protein/min)[a]
Xenotech Male Rat Lung	75.9 ± 3.38[c]	13.7 ± 0.41
F344 Male Rat Lung	144 ± 2.00	18.2 ± 2.85
F344 Female Rat Lung	179 ± 1.53	19.6 ± 2.14
Xenotech Male Mouse Lung	63.7 ± 1.86[d]	5.11 ± 0.36
B6C3F1 Male Mouse Lung	295 ± 29.5	68.9 ± 1.20
B6C3F1 Female Mouse Lung	150[e]	39.8 ± 4.1

[a] Values represent mean ± S.D. (n = 3).
[b] The activity reported by the vendor was 1580 ± 70 pmol/mg protein/min.
[c] The activity reported by the vendor was 70.2 ± 1.5 pmol/mg protein/min.
[d] The activity reported by the vendor was 58.7 ± 1.4 pmol/mg protein/min.
[e] Value is a single determination.

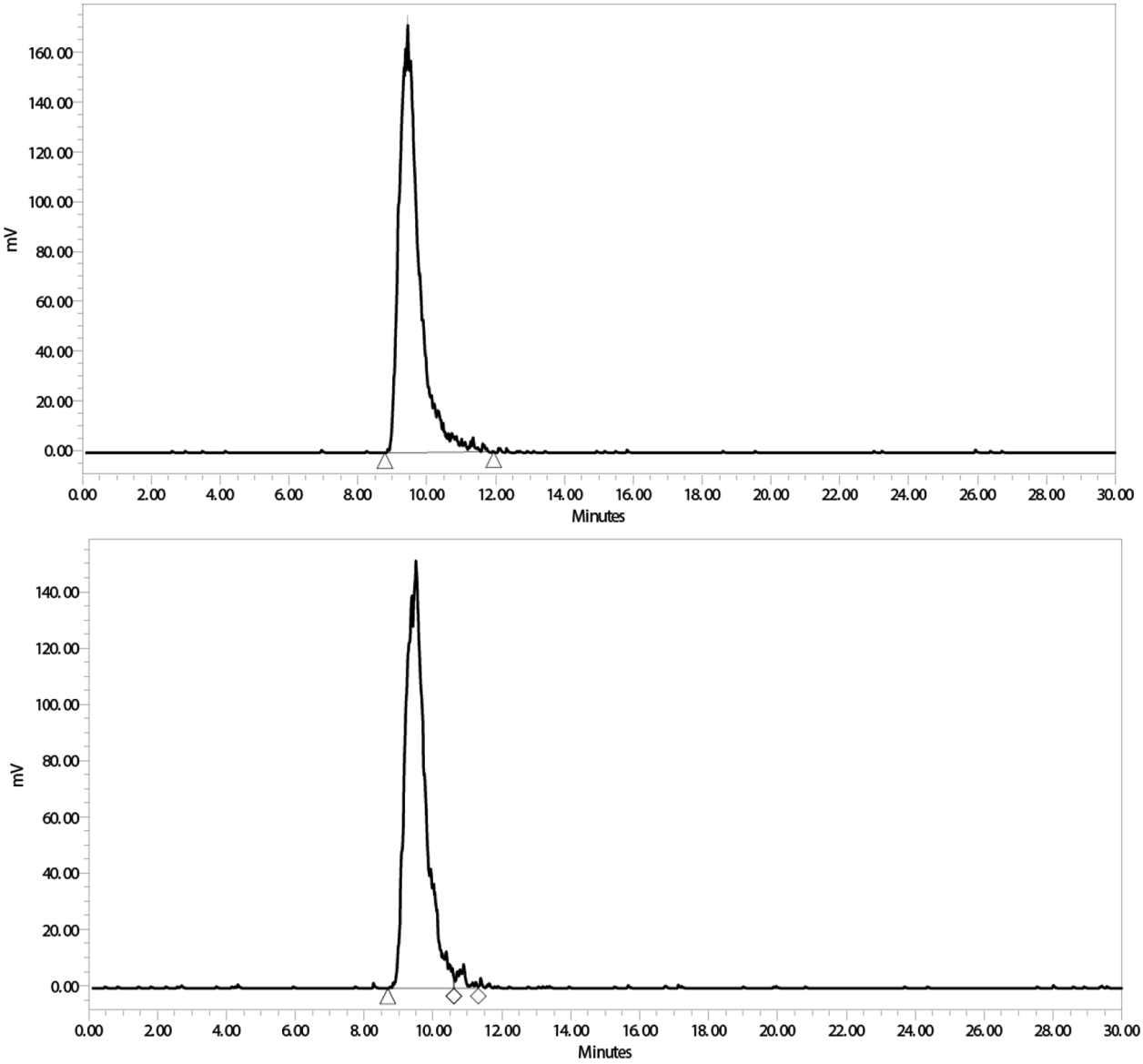

Fig. 3. HPLC radiochromatogram of ^{14}C 4-MeI incubated with male mouse liver microsomes at 0 (top) and 60 min (bottom).

(*m/z* 184 → 120) (Walsky and Obach, 2004). Ethoxycoumarin O-dealkylation was measured by the determination of 7-hydroxycoumarin (*m/z* 160.9 → 132.9). Ethoxyresorufin O-dealkylation was measured by the determination of resorufin (*m/z* 241.1 → 186.1).

For analysis of the potential formation of methylglyoxal, microsomal incubation reactions were quenched by addition of 0.1 volume of 5 M perchloric acid, centrifuged and supernatant removed. To the supernatant 125 nmol o-phenylenediamine was added, mixed and incubated at 4–8 °C for 18–20 h. The samples were then prepared by solid phase extraction and analyzed by LC-MS/MS. Standards of methylglyoxal (1 μM) incubated with microsomes were prepared to verify recovery of methylglyoxal as 2-methylquinoxaline. Analysis was conducted using the method of (Randell et al., 2005) with LC-MS/MS monitoring the MRM 145 → 51.

Incubations with S-9 fractions were conducted in a manner analogous to the incubations conducted for mutagenicity analysis (Beevers and Adamson, 2016), with 4-MeI added at two concentrations of 1.25 and 50 mg/mL. The incubations with S-9 were compared by determining the concentration of 4-MeI after incubation at 37 °C for 20 min relative to time zero. ^{14}C 4-MeI was added to enable tracing the formation of products.

Incubations to determine the metabolism of radiolabeled 4-MeI were conducted with 100 μM [4–^{14}C] 4-MeI and microsomes from male and female rat and mouse lung and liver. After incubation, protein was precipitated by addition of acetonitrile, and the samples chromatographed with detection of radioactivity using a Varian Polaris C18 column, 4.6 × 250 mm (Torrance, Ca) and a binary mobile phase gradient with mobile phase A: 5 mM ammonium hydroxide in water and B: acetonitrile. The initial mobile phase composition of 98:2 A:B was held for 10 min before changing linearly to 80:20 A:B over 5 min and then to 10:90 A:B over 15 min before returning to initial conditions. The flow rate was 1 mL/min and the eluent was monitored for UV absorbance at 220 nm and for radioactivity using a βRAM radioactivity detector with a solid phase cell for rat liver and lung, and with a liquid scintillant cell for mouse liver and lung. FloScint III (Perkin Elmer) was mixed at a rate of 4 mL/min.

2.3.2. Copper ascorbate reaction

4-MeI (100 μM, [4–^{14}C]) was incubated with 50 mM sodium phosphate buffer, 5 mM L-ascorbic acid, and 5 μM cupric chloride for 1, 4, and 24 h at 37 °C.

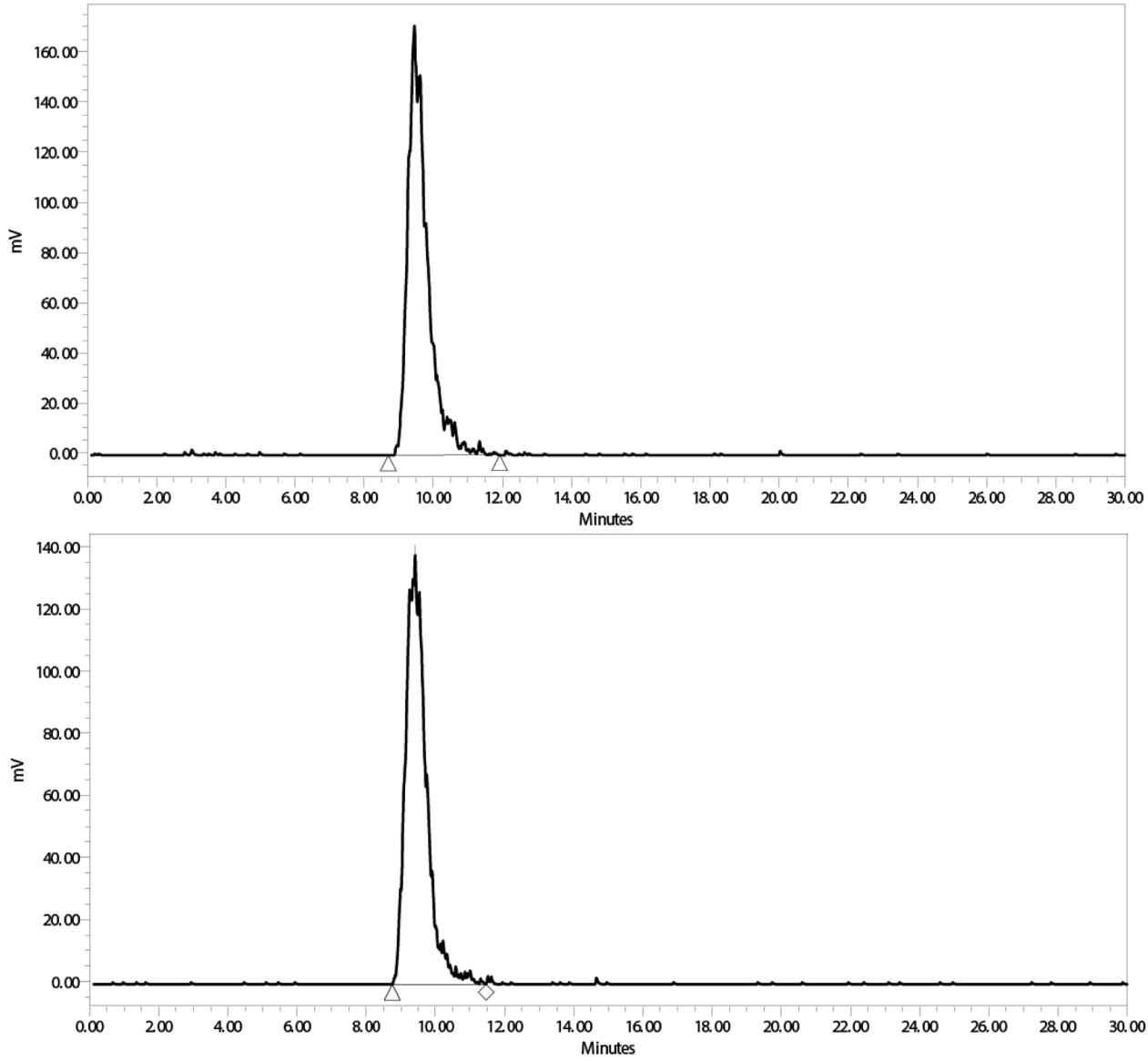

Fig. 4. HPLC radiochromatogram of ^{14}C 4-MeI incubated with male mouse lung microsomes at 0 (top) and 60 min (bottom).

2.4. In vivo administration of [4–^{14}C] 4-MeI

4-MeI was prepared at a specific activity of 0.164 mCi/mmol and 0.0548 mCi/mmol in water at concentrations of 10 and 30 mg/mL, and administered at a dose of 50 or 150 mg/kg, respectively, to male and female F344 rats and B6C3F1 mice. The doses were chosen to correspond to those used by Yuan and Burka (1995) for pharmacokinetic and disposition studies (5, 50 and 150 mg/kg). The doses were also in the range of the daily intake from dietary exposure during the carcinogenesis bioassays (NTP, 2007). Animals were placed in all glass metabolism cages for the collection of urine, and feces. Exhaled air was drawn through two traps containing ethanol and two traps containing 1.0N NaOH for the collection of exhaled volatiles and $^{14}CO_2$. The ethanol traps were cooled on wet ice (first trap) and on dry ice ice/isopropanol (second trap). Trap samples were collected, and trap contents were changed at 1, 2, 4, 8, 24, and 48 h after dosing. After 48 h, animals were euthanized, and liver, kidney, lung, blood and carcass were collected.

Carcass samples were weighed and dissolved in 2 N ethanolic sodium hydroxide. Tissue samples were weighed and solubilized in Soluene-350™. Solubilized tissue and carcass samples were then bleached by adding 125 µL of 70% perchloric acid, followed by 0.3 mL of 30% H_2O_2. Urine samples were analyzed directly by addition of duplicate aliquots into scintillation cocktail. Feces samples were homogenized with an equal volume of water, and then dissolved in Soluene-350™ until dissolved, and then bleached as described above.

Ultima Gold™ scintillation cocktail was added to samples and radioactivity was determined using a Packard 1900CA Liquid Scintillation Counter, using an external standard channels ratio quench correction method. Activities recovered in blood and plasma are expressed per g of sample. Samples were counted for 5 min.

Data for body weights, dosing information including dosing time, sample collection time, sample and aliquot weights and radioactivity were collected using the Debra data collection and reporting system (Lablogic Systems Limited, Sheffield, England).

2.5. HPLC analysis

HPLC analysis of radioactive samples was conducted on system consisting of a Waters (Milford, MA) 600E solvent delivery module, an Applied Biosystems (Foster City, CA) 759A Absorbance detector, and a β-Ram Model 3 flow-through radioactivity detector (IN/US Systems

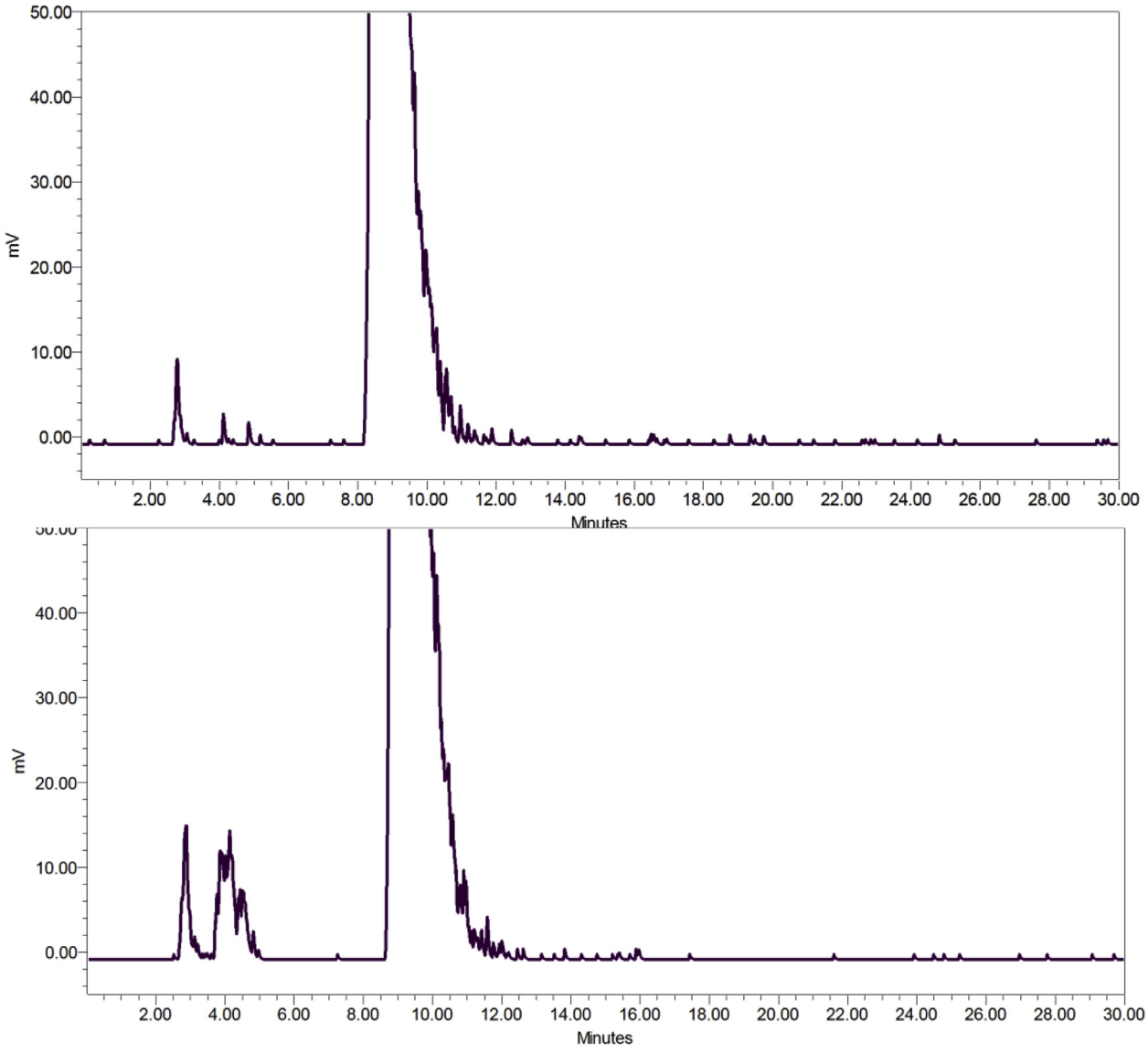

Fig. 5. Radiochromatogram of ^{14}C 4-MeI incubated with (bottom 24 h) or without (top) copper and ascorbate at 37 °C.

Table 2
Recovery of radioactivity (% of dose administered) in rats and mice administered^{14}C 4-MeI.

Dose (mg/kg)	Male Rat	Female Rat	Male Rat	Female Rat	Male Mouse	Female Mouse	Male Mouse	Female Mouse
	50	50	150	150	50	50	150	150
Urine	84.79 ± 7.44	88.69 ± 1.03	83.95 ± 4.61	79.03 ± 5.72	69.77 ± 17.45	48.97 ± 29.09	40.89 ± 33.96	63.94 ± 10.33
Feces	4.23 ± 3.18	1.87 ± 0.42	2.51 ± 1.33	2.43 ± 0.54	4.35 ± 2.44	12.55 ± 9.38	14.54 ± 17.34	13.18 ± 9.03
Cage Rinse	5.45 ± 5.08	3.16 ± 0.59	5.72 ± 2.08	9.48 ± 5.25	18.3 ± 15.94	24.44 ± 14.18	33.56 ± 19.37	14.09 ± 8.35
CO_2	1.72 ± 1.28	1.33 ± 0.24	1.44 ± 0.28	1.66 ± 0.11	1.86 ± 0.48	3.52 ± 1.27	1.57 ± 0.32	1.66 ± 0.75
VOC	0.02 ± 0.03	0.04 ± 0.05	0.10 ± 0.19	0.01 ± 0.01	0.06 ± 0.04	0.08 ± 0.03	0.11 ± 0.05	0.06 ± 0.03
Liver	0.051 ± 0.005	0.054 ± 0.004	0.082 ± 0.01	0.086 ± 0.013	0.067 ± 0.021	0.143 ± 0.072	0.080 ± 0.012	0.074 ± 0.024
Kidney	0.007 ± 0.001	0.007 ± 0.001	0.009 ± 0.001	0.010 ± 0.001	0.018 ± 0.004	0.021 ± 0.012	0.027 ± 0.004	0.011 ± 0.005
Lung	0.003 ± 0.001	0.003 ± 0.000	0.004 ± 0.001	0.005 ± 0.001	0.003 ± 0.001	0.008 ± 0.006	0.004 ± 0.001	0.005 ± 0.002
Carcass	1.93 ± 0.57	1.50 ± 0.38	1.78 ± 0.24	2.02 ± 0.53	1.32 ± 0.71	2.62 ± 2.06	2.16 ± 0.61	1.64 ± 0.44
Total Recovered	98.20 ± 1.34	96.67 ± 0.20	95.61 ± 1.02	94.75 ± 0.83	95.69 ± 2.19	92.20 ± 3.83	92.82 ± 2.96	94.68 ± 2.96

Inc., Tampa, FL). Samples were chromatographed using an Agilent Polaris 5 C18-A column, 4.6 × 250 mm (Santa Clara, CA) using a binary mobile phase gradient with mobile phase A: 0.1% TFA in water and B: 0.1% TFA in acetonitrile. The initial mobile phase composition of 99% A changed linearly to 80% An over 20 min, and then from 80% A to 0% An over the next 10 min before returning to initial conditions. The flow rate was 1 mL/min, and the effluent was monitored for UV absorbance at 210 nm and for radioactivity using an IN/US β-RAM flow-through radioactivity detector equipped with a liquid scintillation cell with FLO SCINT III scintillation cocktail (Perkin Elmer, Waltham, MA) at a flow rate of 1 mL/min.

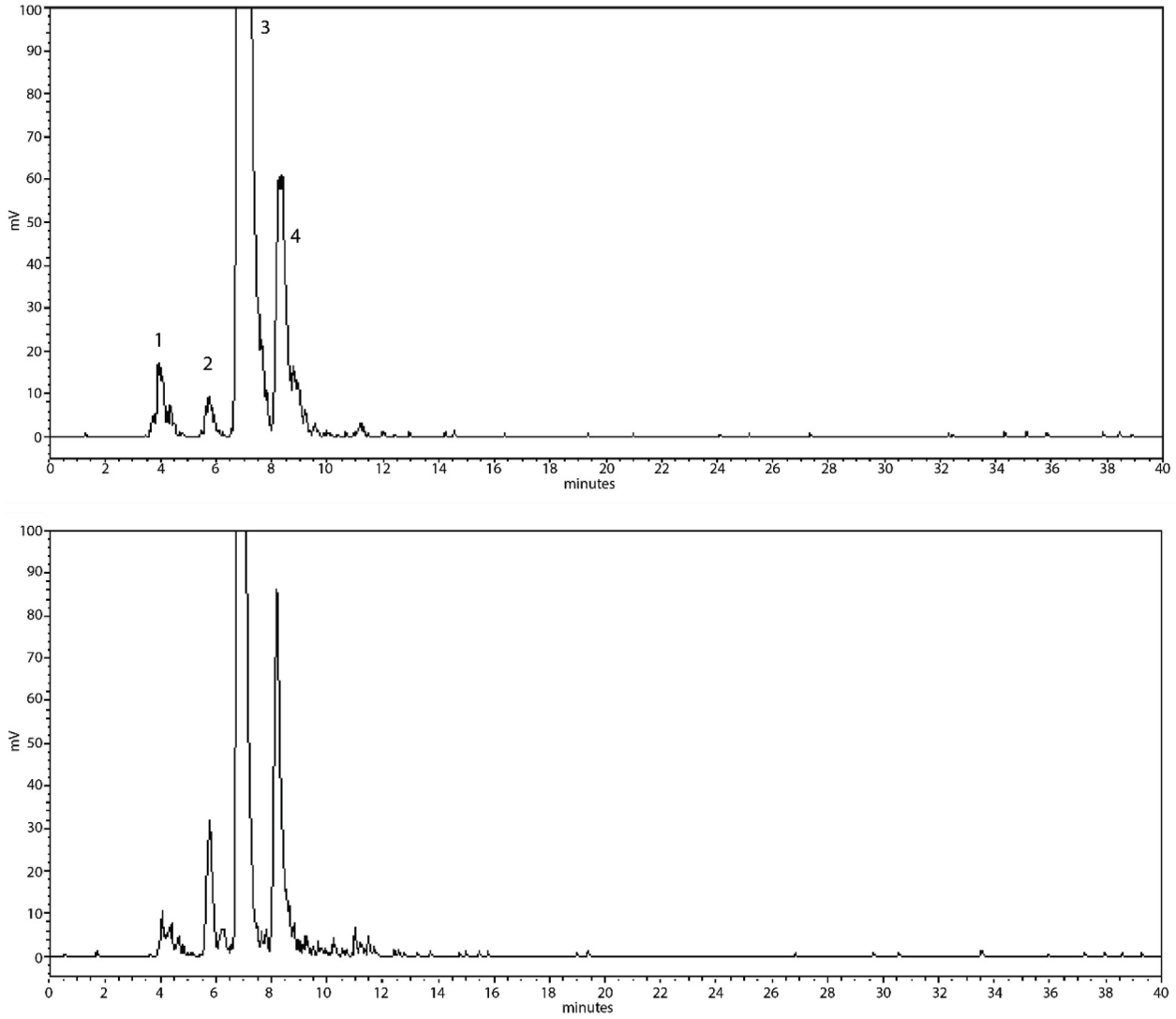

Fig. 6. Radiochromatogram of urine from a male rat (top) and a male mouse (bottom) administered 150 mg/kg ^{14}C 4-MeI.

Table 3
Characterization of radioactivity in urine.

	Dose mg/kg	% of Urinary Radioactivity (Mean ± SD)			
		Peak 1[a]	Peak 2	4-MeI	Peak 4
Male Rat	50	1.92 ± 1.20	0.713 ± 0.169	78.3 ± 2.98	13.7 ± 3.08
	150	3.40 ± 1.35	0.585 ± 0.468	77.2 ± 1.18	11.7 ± 1.64
Female Rat	50	1.52 ± 0.446	0.568 ± 0.519	74.4 ± 3.58	18.1 ± 4.26
	150	3.64 ± 1.30	1.37 ± 1.38	72.9 ± 3.18	17.2 ± 3.05
Male Mouse	50	2.37 ± 1.04	3.79 ± 1.40	70.4 ± 5.22	16.7 ± 5.69
	150	1.72 ± 0.961	3.12 ± 1.24	71.8 ± 5.64	11.1 ± 4.14
Female Mouse	50	4.82 ± 2.46	6.59 ± 5.32	60.1 ± 15.0	20.9 ± 6.45
	150	2.32 ± 1.52	3.05 ± 3.02	76.7 ± 9.15	12.9 ± 4.23

[a] Major component of Peak 1 is identified as 4-hydroxymethylimidazole, for Peak 2 the major component is hydroxymethylimidazole glucuronide, Peak 3 is 4-MeI, and Peak 4 is 4-methylhydantoin.

2.6. LC-MS analysis

Samples of urine and microsomal samples were analyzed by chromatography with an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Framingham, MA) coupled to an Agilent 1100 HPLC system (Santa Clara, CA). Initial scans were conducted in the range of *m/z* 70–500. Microsomal samples were chromatographed using a Varian Polaris C18 column, 4.6 × 250 mm (Torrance, CA) and a binary mobile phase gradient with mobile phase A: 5 mM ammonium hydroxide in water and B: acetonitrile (98:2 A:B for 10 min changing linearly to 80:20 A:B/5 min, and then to 10:90 A:B/15 min before returning to initial conditions. The transition monitored for 4-MeI was 83 → 56. Urine samples were chromatographed using an Agilent Polaris 5 C18-A column, 4.6 × 250 mm (Santa Clara, CA) using a binary mobile phase gradient with mobile phase A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile (100% A changing linearly to 80/20

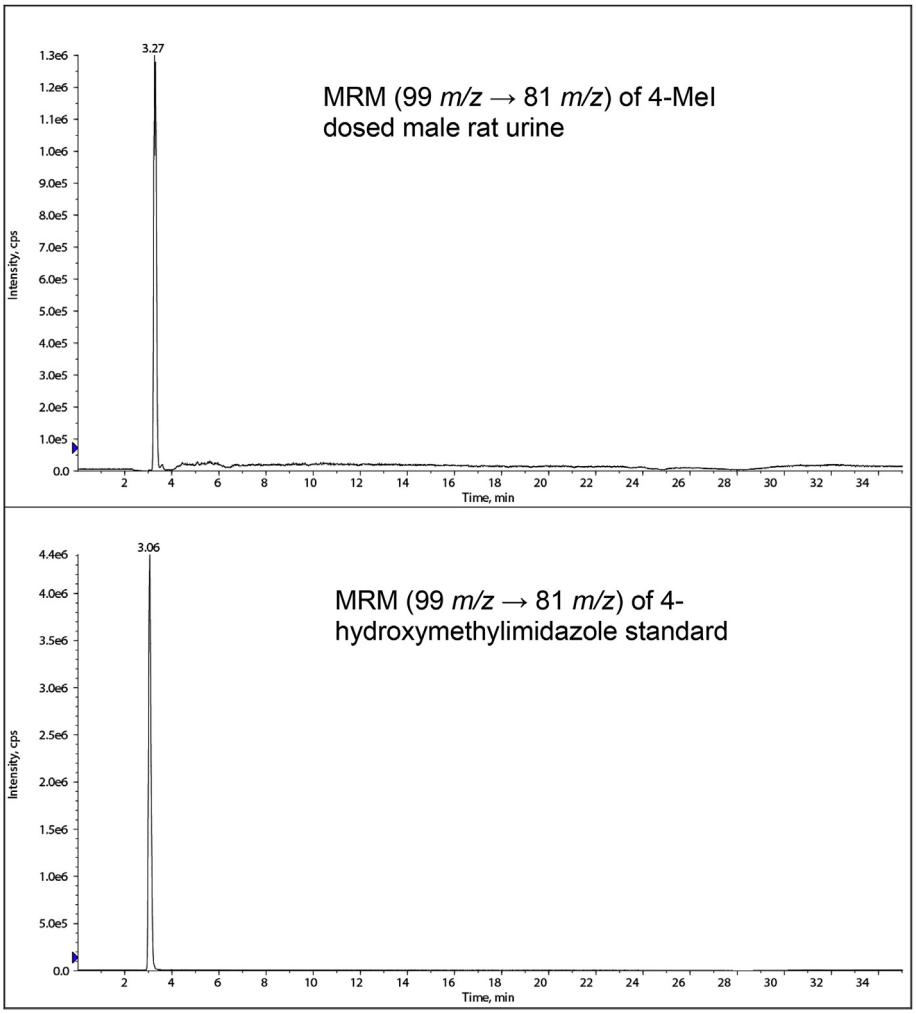

Fig. 7. LC-MS/MS analysis of rat urine indicating the presence of 4-hydroxymethylimidazole (99 → 81) in rat urine (top) and a standard (bottom).

A:B/20 min, and then to 10:90 A:B/5 min before returning to initial conditions). Urine samples for each animal were pooled (5%) and analyzed as one pooled sample per animal.

HPLC analysis of the metabolism of radiolabeled 4-MeI were conducted with 100 μM [4–^{14}C] 4- MeI and microsomes from male and female rat and mouse lung and liver. After incubation, protein was precipitated by addition of acetonitrile, and the samples chromatographed with detection of radioactivity as described above for clearance. Elution (1 mL/min) was monitored for UV absorbance at 220 nm and for radioactivity using a βRAM radioactivity detector. HPLC analysis of the urine samples were chromatographed using the Agilent Polaris column with the same gradient as described for the urine samples above, except TFA was substituted for formic acid.

The characterization of urinary metabolites was conducted on two LC-MS systems, with initial characterization conducted on a triple quadrupole system. In coordination with radioactivity detection, elution of 4-Hydroxymethylimidazole was monitored by LC-MS using a transition of 99 → 83, and comparison with an authentic standard. Other oxidized 4-MeI products were monitored using a product ion scan for *m/z* 99. The presence of 2-methylhydantoic acid was monitored by a transition of 133 → 90, with comparison with an authentic standard. Glucuronides were detected with a neutral loss scan of 176, and the presence of sulfate conjugates with a neutral loss of 80.

A second LC-MS system with high mass resolution detection was conducted using a Waters Acquity UPLC system coupled with a Thermo LTQ Orbitrap Velos. Acquisition was conducted with FTMS in full scan mode with resolution set to 30000. Samples were analyzed in positive ion mode with profile data. The activation type was collision-induced disassociation, with an isolation width of 1.0 m/z. The range of analysis was from 70 to 500 m/z. For each of the postulated metabolites, isotope similarity scores (on a scale from 0 to 100 with 100 being the best possible score) were determined in Progenesis QI, (Non-Linear Dynamics, Durham, NC).

3. Results

3.1. In vitro metabolism and clearance

An LC-MS method for analysis of 4-MeI was developed based on the method of Klejdus et al. (2003). On incubation of 4-MeI with microsomes and NAPDH from mouse and rat lung and liver, there was no significant change in the amount of 4-MeI with time, over a 60-min period. An example chromatogram is shown in Fig. 2. No new peaks appeared. Samples were monitored for expected products based on the addition of oxygen (*m/z* 99). To verify that the microsomal systems were functioning and were capable of oxidation, incubations were conducted with verapamil, propranolol, and chlorzoxazone as Cyp substrates and activity measured for clearance or appearance of product (data not shown). Data for ethoxycoumarin O-dealkylation in liver microsomes, and phenacetin O-dealkylation and ethoxyresorufin O-dealkylation are presented in Table 1. Liver microsomes that were obtained commercially were provided with activity measurements for ethoxycoumarin deethylation, and the values determined in house were similar to those provided by the vendor (1470 vs 1580 pmol/min/mg).

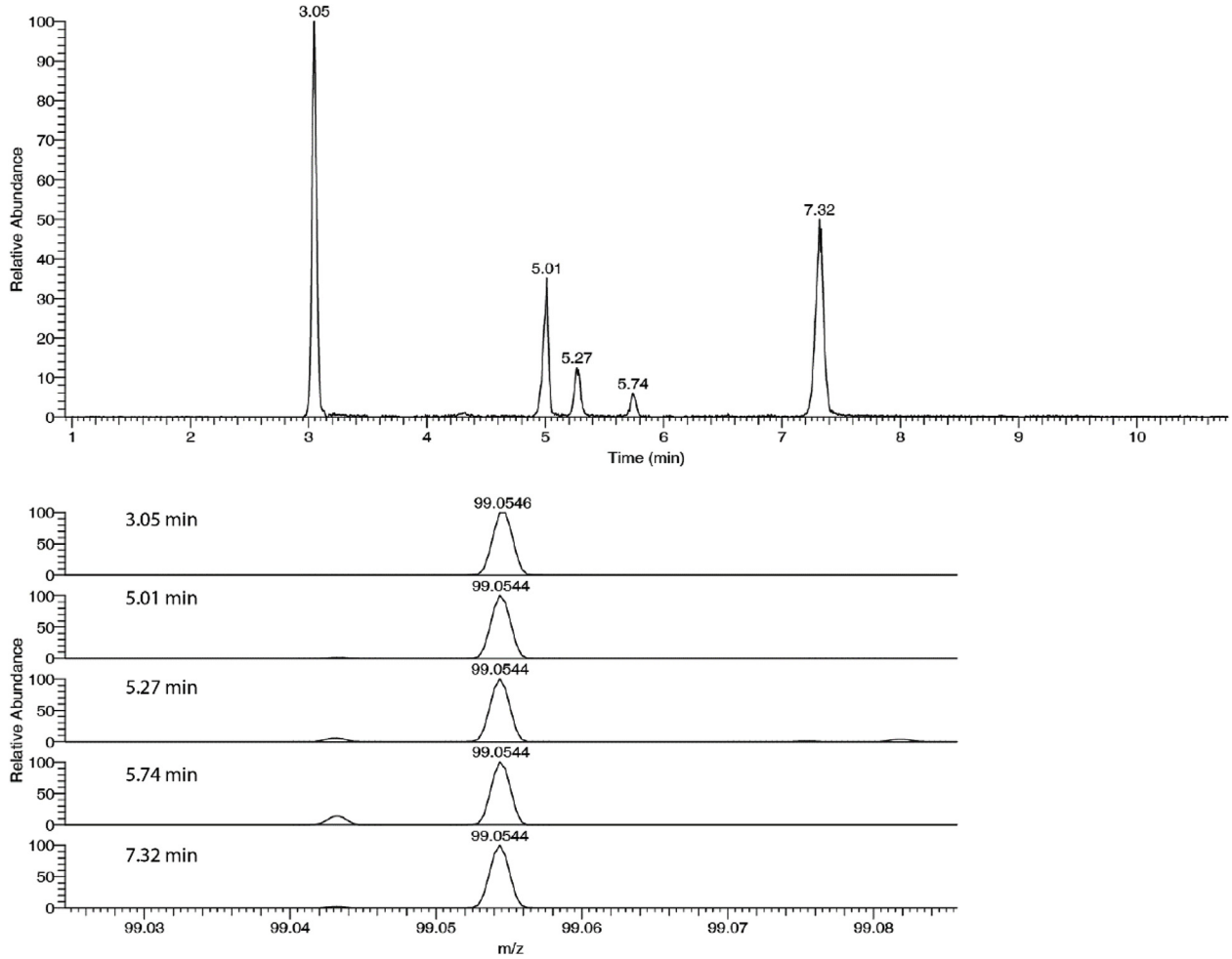

Fig. 8. High resolution LC-MS analysis of male rat urine indicating the presence of 4-hydroxymethylimidazole and additional oxidized methylimidazole metabolites. Top panel shows the chromatogram (*m/z* 99.05–99.06), and the bottom panel indicates the exact mass of the individual peaks at 3.05, 5.01, 5.27, 5.74, and 7.32 min.

In addition, the ethoxycoumarin O-dealkylation activities determined in liver microsomes prepared in house for rats and mice were similar or higher than those in commercially procured microsomes. The activities of determined in house for commercially procured lung microsomes for phenacetin O-dealkylation were similar to the vendor-provided measurements (75.9 vs 70.2 pmol/mg/min for male rat lung, and 63.7 vs 58.7 pmol/mg/min for male mouse lung, Table 1). The activities measured in in-house prepared microsomes were generally higher for rat and mouse lung. These measurements demonstrated that the microsome preparations were metabolically competent with typical CYP substrates.

GSH was added to some incubations as a potential trapping agent for reactive metabolites. No additional peaks were observed that were consistent with metabolites of 4-MeI. Scans were conducted for neutral loss of pyroglutamate (m-129), and for the expected masses for glutathione conjugates (Data not shown).

The in vitro reaction was investigated with ^{14}C 4-MeI as substrate. No new radioactive metabolite peaks were detected in incubations of rat and mouse lung and liver microsomes (Figs. 3 and 4), or with rat and mouse lung and liver S-9. These S9 incubations were conducted to mirror the S-9 incubations used in a recent Ames mutagenicity assay (Beevers and Adamson, 2016). For these S-9 incubations, the concentration of 4-MeI was substantially higher than the previous in vitro assays, and analysis was conducted using HPLC with UV detection of the 4-MeI.

3.2. Assessment of formation of methylglyoxal

It has been proposed that 4-MeI may undergo oxidation to form an epoxide, followed by hydrolysis to form a cyclic hemiaminal that can undergo release of methylglyoxal, a reactive ketoaldehyde. To investigate the formation of methylglyoxal, we used its reaction with o-phenylenediamine to establish whether the methylglyoxal was being formed (Randell et al., 2005). The HPLC analysis indicated that the expected product 2-methylquinoxaline could be readily detected in microsomal samples to which methylglyoxal had been added at a concentration of 1 μM, but not in samples incubated with 4-MeI (data not shown).

3.3. Oxidation with copper and ascorbate

With all of the microsomal systems investigated giving no indication of oxidation products formed, we investigated the feasibility of detection of oxidation products of 4-MeI using a copper ascorbate system that had previously been reported to oxidize 2-methylimidazole (2-MI) (Miyachi and Nagatsu, 2002; Ohta et al., 1998a, 1998b, 1998c). This system had been reported to produce 2-methylimidazolone as an oxidation product of 2-MI. We procured standards of 4-methylhydantoin and 4-hydroxymethylimidazole for use in evaluating the chromatographic system and in comparing with products formed on incubation of 4-MeI with copper and ascorbate. Several radioactive peaks were detected with this system. Incubation of copper and ascorbate with ^{14}C 4-MeI caused an increase in the size of peaks eluting at approximately 3

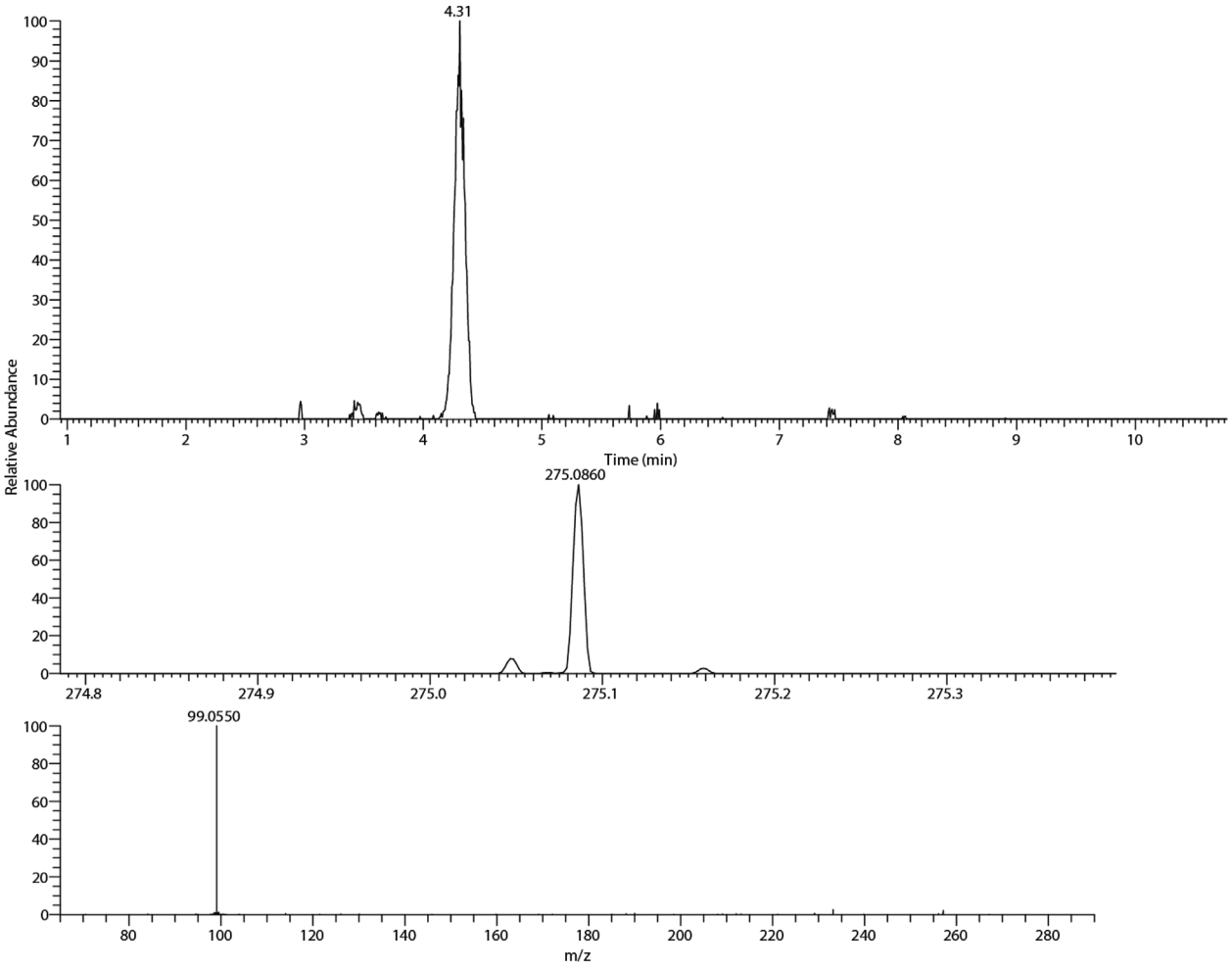

Fig. 9. High resolution LC-MS analysis of rat urine indicating the presence of a glucuronide conjugate of an oxidized methylimidazole (top), the parent ion mass at 275.086, and MS of the parent ion showing a fragment at *m/z* 99.055 by neutral loss of *m/z* 176.

and 4 min (Fig. 5). The percentage of radioactivity converted to products was approximately 3% by 24 h. To verify that oxidation products could be detected if present, we investigated the oxidation of 4-MeI with a model system consisting of copper ions and ascorbate, which has been reported to oxidize 2-MeI.

3.4. *In vivo disposition and metabolism of ^{14}C 4-MeI*

Administration of ^{14}C 4-MeI to male and female rats by gavage at doses of 50 and 150 mg/kg resulted in rapid elimination of radioactivity, primarily in the urine (Table 2). Excretion in feces accounted for 1.9–4.3%. The cage rinse accounted for 3.2–9.5%. A small percentage (1.3–1.7%) was eliminated as $^{14}CO_2$, and almost none (0.01–0.1%) as exhaled volatiles. Between 95 and 98% of the administered radioactivity was recovered from the excreta, tissues and carcass and cage rinse. Radioactivity in the liver was approximately 0.05–0.09% of the dose, in the kidney 0.007–0.010%, and in the lung was 0.003–0.005% of the dose. In the carcass, approximately 1.5–2.0% of the administered dose was recovered.

In mice, 41–70% of the radioactivity was recovered in urine, 4–12% in feces, 18–34% in cage rinse, 1.9–3.5% as CO_2, 1.3–2.6% in carcass, and 0.1% as exhaled volatiles. The substantially lower recovery of radioactivity in the mouse urine compared with rats is offset by a substantially higher recovery in cage rinse. The sum of cage rinse and urine accounted for 74–79% of the dose. The higher recovery of radioactivity in mouse feces may have resulted from urine contamination. Between 92 and 96% of the administered radioactivity was recovered from the excreta, tissues and carcass and cage rinse. Radioactivity in the liver was approximately 0.06–0.14% of the administered dose, in the kidney 0.011–0.027%, and in the lung was 0.003–0.008% of the dose. In the carcass, approximately 1.3–2.6% of the administered dose was recovered.

For the analysis of urinary metabolites, a pooled composite urine sample was prepared for each animal, based on pooling aliquots from each sample based on the proportion of the total urine volume. The composite sample was analyzed by HPLC with determination of radioactivity. Example radiochromatograms from a male rat and male mouse administered 150 mg/kg 4-MeI are show in Fig. 6. The largest radioactive peak was from 4-MeI. Three additional regions of radioactive peaks were observed, and are labeled in order of increasing retention time, peak 1, peak 2, 4-MeI (peak 3) and peak 4.

The majority of the radioactivity eliminated in the urine was in the form of unchanged 4-MeI (Table 3). In rat urine, this accounted for 73–78% of the urinary radioactivity. In mice, this accounted for 60–77%. The group with the lowest mean value for 4-MeI was the female mouse with 60% of the radioactivity in the 4-MeI peak. Peak 4 represented the next largest radioactive peak, and accounted for 12–17% in rats, and 11–21% in mice, with female mice at 50 mg/kg representing the largest amount.

The largest peak (peak 3) was confirmed as 4-methylimidazole by LC-MS by comigration and fragmentation that was similar to a standard (not shown). The earliest eluting peak (Peak 1) was characterized by comigration with a standard and by LC-MS/MS as 4-hydroxymethylimidazole. LC-MS/MS chromatograms for the MRM at *m/z*

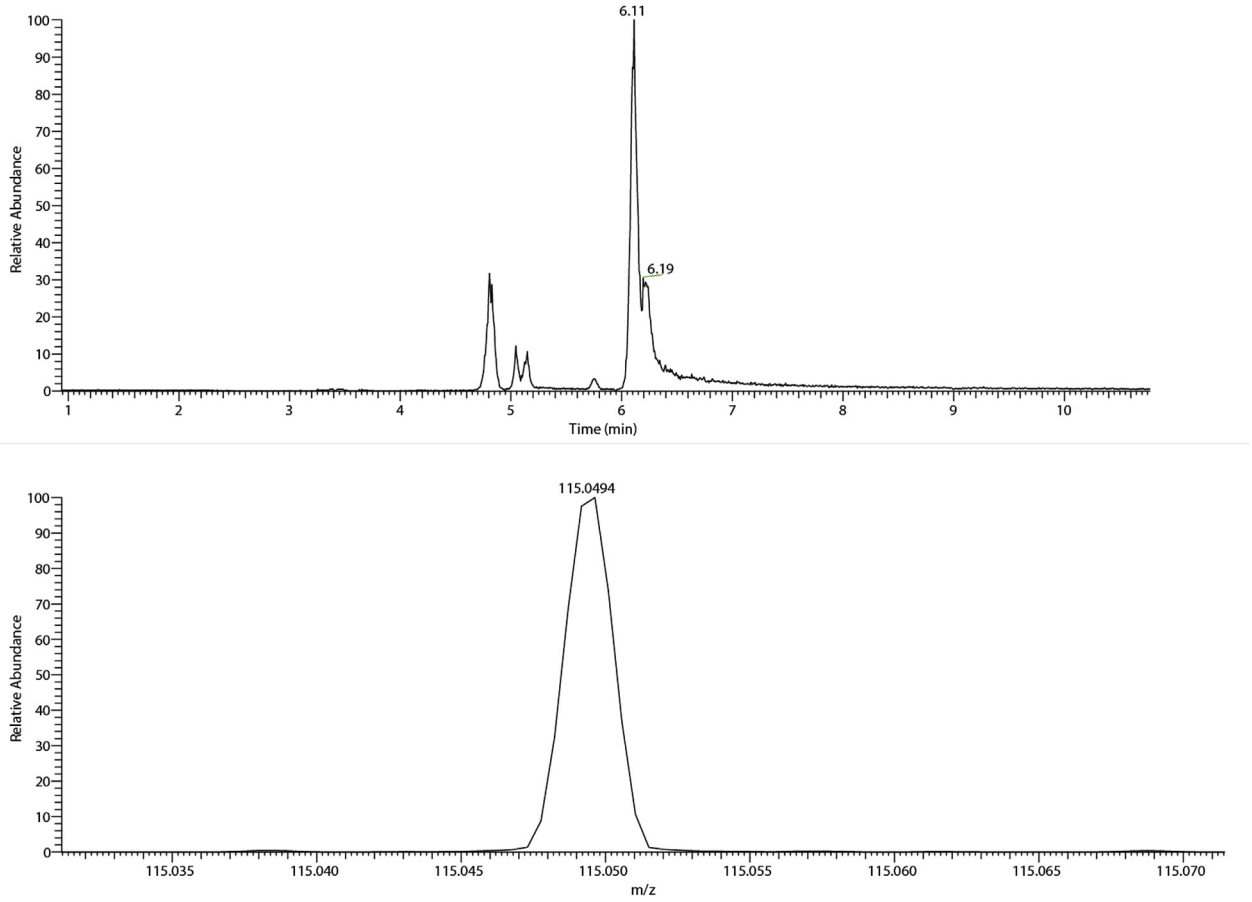

Fig. 10. High resolution LC-MS analysis of male rat urine indicating the presence of a 4-methylhydantoin. Top panel shows the chromatogram (*m/z* 115.04–115.05), and the bottom panel indicates the exact mass of the individual peak at 6.11 min.

99 → 81 for 4-hydroxymethylimidazole are shown in Fig. 7, with a urinary metabolite compared with the authentic standard. Product ion scans at *m/z* 99 indicated the presence of two other peaks at 5.68 and 8.04 min (not shown). Additional examination of the formation of oxidized products was obtained by LC with high resolution mass spectrometry. Five peaks not present in the predose urine were detected with exact mass of *m/z* 99.0544–99.0546 (Fig. 8). The first of these peaks had the same retention time as 4-hydroxymethylimidazole resulting from oxidation of the methyl group. Each had an isotope peak at *m/z* 100.0586 (vs 100.0592 as the expected mass for the M + 1 peak for $C_4N_2H_6O + H^+$) and in Progenesis software, gave isotope similarity scores ranging from 94.8 to 97.7. The 4 additional peaks provided evidence of ring hydroxylation and could be consistent with oxidation of the carbon atoms at the 2 and 5 positions, or oxidation of the nitrogens at the 1 and 3 positions. Also isomeric with this, although not expected to be stable, would be an epoxide. With the exception of the first peak assigned to 4-hydroxymethylimidazole, these hydroxylated metabolites were not resolved from the radioactive peak of 4-MeI.

Evaluation of urine samples for the presence of conjugates of methylimidazole or glucuronide of 4-hydroxymethylimidazole was conducted by scanning for neutral loss of 176 for glucuronides, and comparing the chromatograms from predose urine samples with those from a dosed rat urine sample (not shown). A peak at 4.53 min in the chromatogram of dosed rat urine gave a spectrum with a peak at *m/z* 275, consistent with the expected spectrum for hydroxymethylimidazole glucuronide. A product ion scan at *m/z* 275 (not shown) indicated the presence of a peak at 4.59 min, which produced a fragment also consistent with hydroxymethylimidazole glucuronide. Chromatography with exact mass detection produced additional evidence for the formation of glucuronides with the detection of a broad peak at 4.31 min with *m/z* 275.0860 (Fig. 9), an M + 1 isotope peak at 276.0892, and an isotope similarity score of 95.54. A single fragment ion at *m/z* 99.0550 was observed, consistent with the formation of hydroxymethylimidazole glucuronide. The glucuronide eluted with a similar retention time as the major peak identified as 4-MeI, and thus is not quantitated separately (see Fig. 9).

The presence of 4-methylhydantoin was established by high mass resolution LC-MS, with the detection of a peaks at 6.11 min, with an exact mass of 115.0494 (see Fig. 10). The peak had the same retention time and mass as an authentic standard.

In mouse urine, similar metabolites were detected by LC-MS. The presence of hydroxylated metabolites was confirmed, with one fewer metabolite than in rat urine (Fig. 11). Three of these peaks aligned with three of the rat urine metabolites. However, one of these with a retention time of 5.50 min appeared to be different from the rat.

4. Discussion

While the carcinogenic effects of 4-MeI have been investigated in male and female rats and mice (NTP, 2004), the metabolism and disposition of 4-MeI in vivo has previously been conducted in the male rat only (Yuan and Burka, 1995). No investigations of the metabolism of 4-MeI in vitro have been reported. To understand factors that may contribute to the species difference in response, this study investigated metabolism in vitro in rats and mice, and compared with metabolism in vivo. The potential oxidative metabolism of 4-MeI in lung and liver microsomes from male and female rats and mice at concentrations of 1, 10 and 100 μM. No significant clearance was detected by quantitative analysis of 4-MeI by LC-MS. The ability of the microsomal systems to metabolize several Cyp substrates was verified, and data for

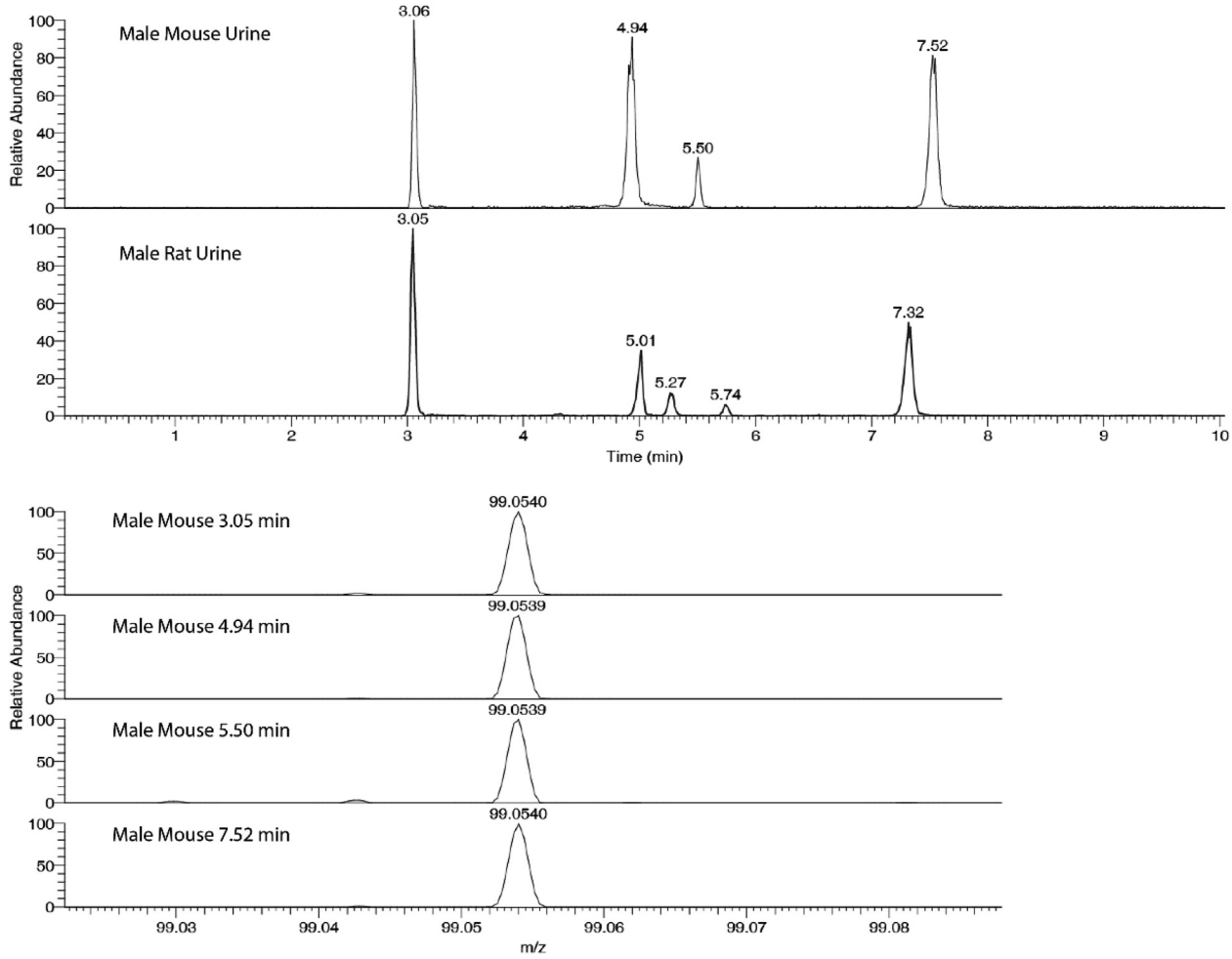

Fig. 11. High resolution LC-MS analysis of male mouse and male rat urine indicating the presence of 4-hydroxymethylimidazole and additional oxidized methylimidazole metabolites. Top panel shows the chromatograms (*m/z* 99.05–99.06) with male mouse urine on top, and the bottom panel indicates the exact mass of the individual peaks from mouse urine at 3.05, 4.94, 5.5, and 7.52 min.

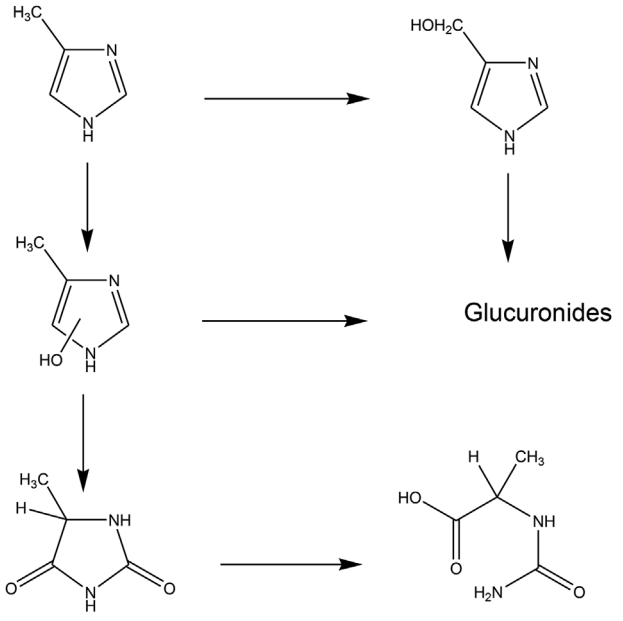

Fig. 12. Metabolism of 4-MeI in vivo.

ethoxycoumarin O-deethylase are reported, as a substrate that indicates activity with variety of Cyp isoforms, including Cyps 2E1, 1A1, 1A2, 2A6, and 2B6 (Ryan and Levin, 1990; Yang et al., 1999). The data obtained in this study compared well with vendor reported activity for male rat liver microsomes, and similar activities for rat and mouse liver microsomes. No clearance of 4-MeI in S9 from mouse lung, mouse liver, rat lung and rat liver was detected. In addition, radiolabeled 4-MeI was incubated with microsomes to evaluate the possible formation of metabolites in vitro. No new radiolabeled products were observed in these microsomal incubations. The inhibition of CYP activity by 4-MeI has been described in vitro and in vivo as noted in the Introduction. Compared with other methylimidazole isomers, 4-MeI has a substantially lower Ki for p-nitrophenol hydroxylase activity at 23 μM, compared with 2700 and 11,000 μM for 1-methylimidazole and 2-methylimidazole, respectively (Hargreaves et al., 1994). Tolbutamide 4-hydroxylase was inhibited in vitro in human liver microsomes by 4-MeI in a non-competitive manner with a Ki of 40 μM (Back et al., 1988). Therefore, it is possible that 4-MeI in vitro is inhibiting CYP activity rather than acting as a substrate. An alternative system consisting of copper ascorbate capable of oxidizing 2-MeI has been reported (Miyachi and Nagatsu, 2002; Ohta et al., 1998a, 1998b; 1998c). This system was used to verify that the systems used for separation of metabolites would be capable of detecting the products if 4-MeI was oxidized.

In vivo in rats administered ^{14}C 4-MeI, radioactivity was rapidly eliminated, with the majority of the radioactivity excreted unchanged

in urine. This observation was consistent with the report of extensive renal clearance as the route of elimination of 4-MeI in the male rat (Yuan and Burka, 1995).

Characterization of the urinary metabolites detected indicated the formation of hydroxylated metabolites, identified as 4-hydroxymethylimidazole, as well as ring oxidized products with oxidation potentially at all positions, and a glucuronide (Fig. 12).

While multiple metabolites were detected here that were consistent with the formation of products with a single oxidation, with the exception of 4-hydroxymethylimidazole, the site of oxidation is not known. With the formation of 4-methylhydantoin indicating oxidation at each of the ring carbons, oxidation individually at each of the ring carbon atoms in 4-MeI is probable. With the presence of unsaturation in the ring, rearrangement to the keto conformation would be expected. The presence of multiple peaks isomeric peaks with the same exact mass, and the possible difference between rats (5 peaks) and mice (4 peaks) with three overlapping peaks, two unique to rats and one unique to mice points to some small differences in metabolism. While the mono-oxidation at all possible sites in the ring and on the methyl group could account for 5 metabolites, it appears that there are 6 metabolites with this molecular formula. However at this time, since the isomers all have the same exact mass, retention time drift between the various samples has not been eliminated. An epoxide is a potential isomer that would have the same molecular formula. The reactivity of the potential oxidation products is not known, however some have been synthesized or isolated as natural products and reported in the literature or in patents; e.g. 4-methyl-4-imidazolin-2-one, (Andrews, 1986), 4-methyl-2-imidazolin-4-one (He et al., 2014), and compounds with oxidation on the imidazole nitrogens have been described (Eriksen et al., 2001; Volodarskii et al., 1980).

One of the challenges in using LC-MS for analysis of the 4-MeI and its metabolites is the difficulty in retaining the polar metabolites on the column and finding conditions that would give good resolution of metabolite peaks and good mass spectral response. While an ammonia containing mobile phase worked well for 4-MeI chromatography, this was not optimal for metabolite resolution. While metabolite peaks could be resolved well for detection of radioactivity with TFA in the mobile phase, this was not optimal for detection by LC-MS. A combination of LC-MS/MS with a triple quadrupole mass spectrometer and high resolution LC-MS and LS-MS/MS with an Orbitrap mass spectrometer was used to ensure that the maximum number of metabolites could be identified.

The treatment of hay with ammonia to increase digestibility and nitrogen content has led to intoxication of livestock. The identification of 4-MeI as a by-product of this process led to the investigation of the metabolism of 4-MeI in goats and heifers (Nielsen et al., 1993). 2-[^{14}C] 4-MeI was administered to two lactating goats at dose of 20 mg/kg by intravenous and oral injection. 67% (IV) and 64% (oral) of the radioactivity was excreted in urine with 22% (IV) and 16% (oral) as unchanged 4-MeI, 40% (both routes) as 5-methylhydantoin, 18% (both routes) as 2-methylhydantoic acid, and 10 (IV) and 14% (oral) as an unidentified metabolite. Two heifers received 2-[^{14}C] 4-MeI administered at a dose of 20 mg/kg by intravenous injection. 92% of the radioactivity was excreted in urine with 75% as unchanged 4-MeI, 11% as 5-methylhydantoin, 4% as 2-methylhydantoic acid, and 7% as an unidentified metabolite. ^{14}C labeled urea was identified in urine from goats at approximately 1–2%. The identification of 2-methylhydantoic acid indicated that 4-MeI could undergo metabolism resulting in ring opening in these species.

The results reported in goats and heifers differ dramatically from the results reported here for rats and mice. To identify the specific oxidation products, we determined the formation of 5-methylhydantoin and methylhydantoic acid. Methylhydantoin was detected in urine by LC-MS/MS both in the predose urine samples and following administration of 4-MeI, but did not appear to increase as a result of MeI administration. 2-Methylhydantoic acid was detected in urine of rat and mice prior to dosing and following dosing. There appeared to be a small increase in 2-methylhydantoic acid with the dose of 4-MeI administered. However, the recovery of 2-methylhydantoic acid was consistent with approximately 1% or less of the urinary radioactivity.

While CYP enzymes do not appear to play a role in the metabolism of 4-MeI based on in vitro studies, the presence of metabolites in urine indicates that some enzyme systems are capable of metabolizing 4-MeI by oxidation. Based on the literature, there are several candidate enzymes that might capable and might be involved (Garattini et al., 2003, 2009; Garattini and Terao, 2012; Strolin Benedetti, 2011; Strolin Benedetti et al., 2006), which will form the basis of future research. Flavin-containing monoxygenases (FMOs) mainly catalyze the oxidation of nitrogen- and sulfur-containing compounds. FMOs are expressed in a number of forms, and are expressed in a variety of tissues, including lung, liver, kidney and intestine. Molybdenum-containing hydrolases include aldehyde oxidase and xanthine oxidase. Aldehyde oxidase is extensively involved in the oxidation of carbon adjacent to nitrogen in heterocyclic rings (Kitamura et al., 2006). The metabolism of 4-methylpyrazole, an inhibitor of alcohol metabolism by competitive inhibition of alcohol dehydrogenase, involves the metabolism via oxidation of the methyl group to 4-hydroxymethylpyrazole, and further oxidation to 4-carboxypyrazole (Blomstrand and Ohman, 1973; Murphy and Watkins, 1972).

This investigation included the investigation of any metabolism of 4-MeI under the conditions used in the determination of mutagenic activity in vitro (Beevers and Adamson, 2016), and did not detect any metabolism in vitro, yet metabolism is clearly evident in vivo. This suggests that rat or mouse S-9 may not be capable of metabolically activating 4-MeI in mutagenicity assays in vitro, and additional steps may be necessary for the assessment of mutagenicity in vitro (Ku et al., 2007). While a recent report of a positive assay for chromosome aberrations in mice administered 4-MeI (Norizadeh Tazehkand et al., 2016) requires further investigation, the weight of evidence does not support genotoxicity. In reviewing compounds that are negative for genotoxicity in vitro, but positive in vivo (Tweats et al., 2007), one of several explanations proposed was differences between metabolism in vitro and in vivo.

In conclusion, 4-MeI was excreted in urine largely unchanged in male and female mice and rats (between 60 and 78% if the urinary radioactivity). Metabolites were not detected in *in vitro* studies. However, in *in vivo* studies, several metabolites were detected, including hydroxylated 4-MeI and glucuronides that accounted for 22–40% of the urinary radioactivity. There did not appear to be substantial differences in metabolism or clearance of 4-MeI between mice and rats that could account for the differences reported in the carcinogenic activity of 4-MeI in mice but not rats (NTP, 2007).

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.10.032.

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