0090-9556/06/3412-2036–2043$20.00
DRUG METABOLISM AND DISPOSITION
Vol. 34, No. 12
12104/3154052
DMD 34:2036–2043, 2006
Printed in U.S.A.

In Vivo Metabolism of L-Methionine in Mice: Evidence for Stereoselective Formation of Methionine-*d*-Sulfoxide and Quantitation of Other Major Metabolites

Joseph T. Dever and Adnan A. Elfarra

Department of Comparative Biosciences, and Molecular and Environmental Toxicology Center, University of Wisconsin-Madison, Madison, Wisconsin

Received July 18, 2006; accepted September 8, 2006

ABSTRACT:

Flavin-containing monooxygenases (FMOs) 1–4 oxidize methionine (Met) to methionine sulfoxide (MetO). FMO3, the primary isoform expressed in adult human liver, has the lowest K_m and favors methionine-*d*-sulfoxide (Met-d-O) formation over methionine-*l*-sulfoxide. Because female mice, but not males, also express FMO3 in liver, levels of Met and its major metabolites were determined in male or female mice dosed with 400 mg/kg Met i.p. The results show that Met levels in male and female mouse liver or plasma increased significantly at both 15 and 30 min after the Met treatment; Met plasma and liver levels at 30 min were similar to or lower than the corresponding levels at 15 min. Liver and plasma MetO levels increased significantly in both sexes at 30 min, and Met-d-O was the major MetO diastereomer detected. Interestingly, less than 0.1% of the Met dose was excreted in the urine (0–24 h) as Met and Met-d-O. *S*-Adenosylmethionine (SAM) was the major metabolite detected in liver at 15 min. Liver SAM levels at 30 min were lower than the levels at 15 min, and the plasma SAM levels at both 15 and 30 min were much lower than the corresponding levels in the liver. Increases in liver and/or plasma *S*-adenosyl-L-homocysteine, 5′-deoxy-5′-(methylthio)adenosine, and *N*-acetyl-L-methionine were also detected. Taken together, these results suggest that mice extensively and rapidly used the Met dose. Although mice exhibited increases in tissue MetO levels, a major role for FMO3 in Met-d-O formation is not certain since the MetO increases were mostly similar in both males and females.

Flavin-containing monooxygenases (FMOs) are microsomal enzymes that catalyze the NADPH- and O_2-dependent oxidation of heteroatoms (nitrogen, sulfur, phosphorus) present in the chemical structure of a variety of drugs and xenobiotics. Five functional forms (FMO1–5) have been characterized to date (Ziegler, 2002). In humans, FMO1 is the primary isoform expressed in neonate liver, but a switch occurs shortly after birth to FMO3, the primary isoform expressed in adult human liver (Koukouritaki et al., 2002). In mice, females, but not males, also express FMO3 in the liver (Falls et al., 1995; Ripp et al., 1999b). This sex-specific expression of FMO3 in mice makes them an attractive model for studying the role of FMO3 in human drug metabolism and disease.

Our laboratory first identified Met as a substrate for cDNA-expressed rabbit FMO1–3 with K_m values of 48.0, 29.9, and 6.5 mM, respectively (Duescher et al., 1994). The *V*/*K* values (0.9, 1.7, and 6.1 for FMO1–3, respectively) also suggested that FMO3 was the most efficient Met *S*-oxidizer of these three FMO isoforms. FMO3 *S*-oxidation of Met was highly stereoselective, forming 8.4 times more methionine-*d*-sulfoxide (Met-d-O) than methionine-*l*-sulfoxide (Met-l-O), whereas the *d*:*l* diastereomeric ratios for FMO1 and FMO2 were 1.5:1 and 0.7:1, respectively; FMO5 *S*-oxidation of Met was not detected. Recombinant human FMO3 exhibited a K_m value of 3.7 mM with a *V*/*K* value of 4.6 and resulted in stereoselective formation of Met-d-O (90–95%) (Ripp et al., 1999b). Recombinant human FMO4 *S*-oxidation of Met exhibited a K_m value greater than 10 mM with only 30% of the total sulfoxide formed being Met-d-O (Ripp et al., 1999a). The *V*/*K* value for FMO4 was not determined. These data indicated that FMO3 *S*-oxidation of Met proceeds with the highest affinity and greatest diastereomeric selectivity among FMO1–5.

Stereoselective formation of Met-d-O was also detected in rabbit and rat liver microsomes incubated with Met, and exhibited K_m and *V*/*K* values similar to those of cDNA-expressed FMO3 (Duescher et al., 1994; Krause et al., 1996). The latter results provided evidence for FMO3 being the primary isoform involved in Met *S*-oxidation in rabbit and rat liver. Additional experiments also provided evidence that FMOs, but not cytochrome P450s, peroxidases, or reactive oxygen species, mediated the Met *S*-oxidase activity (Krause et al., 1996).

This research is supported by National Institutes of Health Grants R01 DK44295 and T32-ES-007015. A preliminary report of these data was presented at the Federation of the American Societies for Experimental Biology meeting held in San Francisco, CA on April 1–5, 2006 (Dever and Elfarra, 2006).

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.106.012104.

ABBREVIATIONS: FMO, flavin-containing monooxygenase; Met, L-methionine; Met-d-O, methionine-*d*-sulfoxide; Met-l-O, methionine-*l*-sulfoxide; Met-dl-O, L-methionine-*dl*-sulfoxide; NAM, *N*-acetyl-L-methionine; NAMO, *N*-acetyl-L-methionine-*dl*-sulfoxide; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; MTA, 5′-deoxy-5′-(methylthio)adenosine; 3-MTP, 3-methylthiopropionic acid; 3-MTPO, 3-(methylsulfinyl)propionic acid; KMTB, 2-keto-4-methylthiobutyrate; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; ACN, acetonitrile; PBP, *p*-bromophenacyl bromide; MAT1A, methionine adenosyltransferase 1A; 4-HPPD, 4-hydroxyphenylpyruvate dioxygenase.

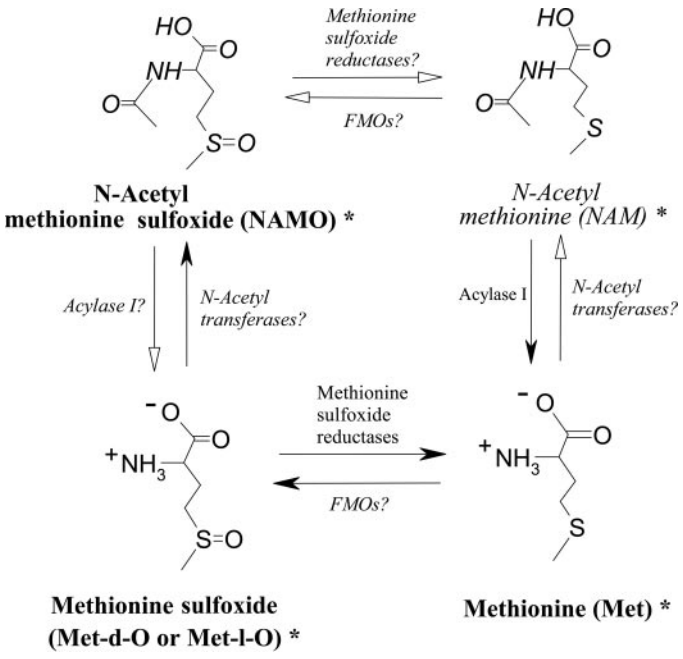

FIG. 1. Proposed *S*-oxidation and *N*-acetylation pathways of Met. Solid arrows indicate known in vivo pathways. Open arrows indicate hypothesized in vivo pathways. Boldface metabolites have been previously detected in vivo. Methods were developed for detection and quantitation of asterisked metabolites.

Human liver microsomes also had high Met *S*-oxidase activity, Met-d-O being the major diastereomer formed (Ripp et al., 1999a,b). Female mouse liver microsomes, which showed Met *S*-oxidase activity similar to that of male and female human liver microsomes, exhibited nearly 3-fold higher activity than male mouse liver microsomes. These results indicated that FMO3 plays an important role in Met *S*-oxidation in female mouse and male and female human liver microsomes when Met is present in the millimolar range. Such a range is significantly higher than normal free Met plasma concentrations in healthy humans (40–60 μM). However, homocystinuric children, patients with alcoholic liver disease, and humans with defects in the Met transmethylation and trans-sulfuration pathway may exhibit plasma Met levels from 0.1 to 1.9 mM (Gahl et al., 1988; Blom et al., 1989; Tangerman et al., 2000; Finkelstein, 2003; Mashima et al., 2003). Liver Met levels were not measured directly in these cases, but such high plasma Met concentrations may indicate high liver Met levels as well. Because high levels of Met have been implicated in cholestasis, cirrhosis, aminoacidemia, hypoglycemia, atherogenesis, and/or death (Shinozuka et al., 1971; Regina et al., 1993; Moss et al., 1999; Halsted et al., 2002; Zhang et al., 2004), FMO3 *S*-oxidation of Met may be important in overall Met metabolism and toxicity.

The primary goal of this study was to quantitate MetO formation in the liver, plasma, and urine of male and female mice dosed with 400 mg/kg Met. This dose was selected to raise plasma and liver Met concentrations to levels seen in some humans with impaired Met metabolism. Liver, plasma, and urine levels of potential Met metabolites in several Met metabolic pathways including the Met *N*-acetylation, transmethylation, and transamination pathways (Figs. 1, 2 and 3, respectively) were also determined to assess the relative roles of these pathways in the metabolism of a single high dose of Met.

Materials and Methods

Chemicals. Met, L-methionine-*dl*-sulfoxide (Met-dl-O), *N*-acetyl-L-methionine (NAM), *S*-adenosyl-L-methionine (SAM), *S*-adenosyl-L-homocysteine (SAH), 5′-deoxy-5′-(methylthio)adenosine (MTA), *S*-methyl-L-cysteine, and trifluoroacetic acid (TFA) were obtained from Sigma Chemical Co. (St. Louis, MO). *N*-Acetyl-L-leucine was purchased from Acros Organics (Fairlawn, NJ). *N*-Acetyl-L-methionine-*dl*-sulfoxide (NAMO) was purchased from Chem-Impex International (Wood Dale, IL). 3-Methylthiopropionic acid (3-MTP) was purchased from Lancaster (Pelham, NH). *p*-Bromophenacyl bromide (PBP) and 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) were obtained from Pierce Chemical Co. Inc. (Rockford, IL). HPLC-grade acetonitrile (ACN) and methanol were obtained from EM Scientific (Gibbstown, NJ). All other chemicals and reagents were of the highest quality commercially available.

3-(Methylsulfinyl)propionic acid (3-MTPO) was synthesized by a method adapted from the synthesis of *S*-allyl-L-cysteine sulfoxide (Ripp et al., 1997). In brief, 50 mg of 3-MTP was dissolved in 2 ml of H_2O and 40 μl of 35% H_2O_2

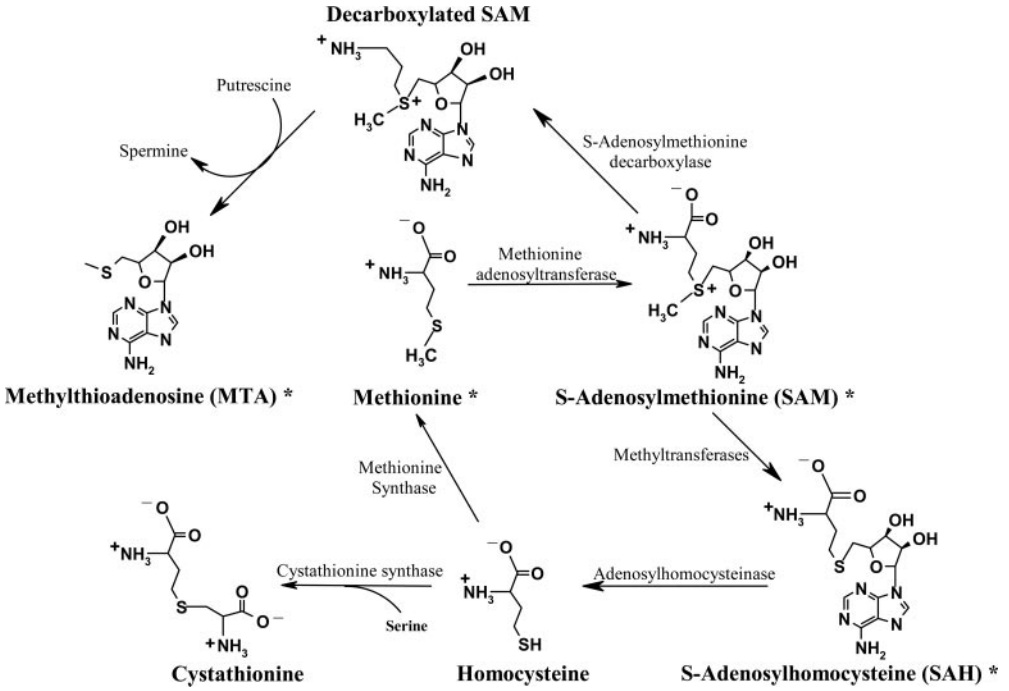

FIG. 2. Met transmethylation and trans-sulfuration pathways. Methods were developed for detection and quantitation of asterisked metabolites.

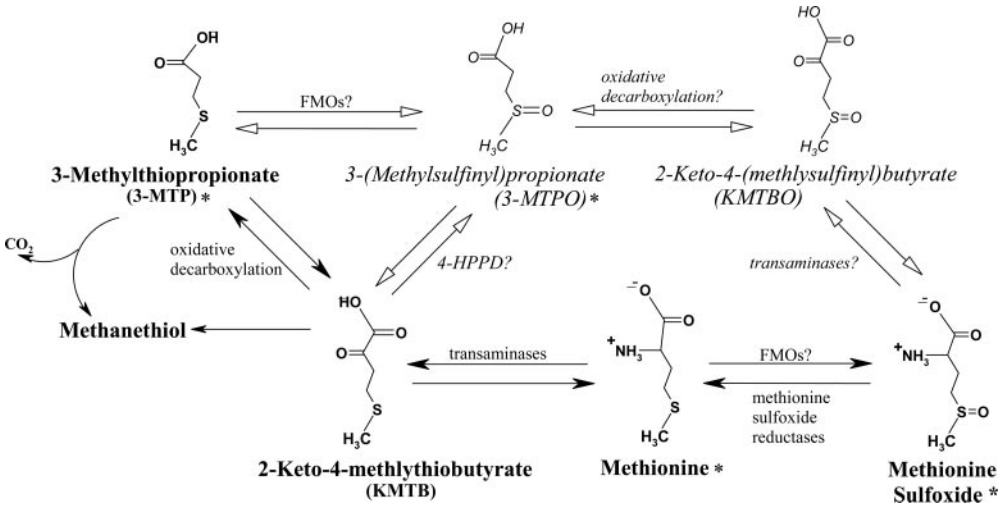

FIG. 3. Proposed transamination pathways of Met. Solid arrows indicate known in vivo pathways. Open arrows indicate hypothesized in vivo pathways. Boldface metabolites have been previously detected in vivo. Methods were developed for detection and quantitation of asterisked metabolites.

was added. The solution was stirred at room temperature for 3 h. Unreacted 3-MTP was removed via diethyl ether extraction (three times, 4 ml). The product was recovered after drying the product solution in a Thermo SPD111V SpeedVac (Thermo Electron Corporation, Waltham, MA). The reaction yield was 90% and the product was >95% pure as determined by HPLC with detection at 220 nm. Identity of 3-MTPO was confirmed by ^{1}H NMR using D_2O as the solvent and was consistent with previously reported ^{1}H NMR spectral data (Doi et al., 1986).

Animals. Male and female B6C3F1 mice (18–25 g) were purchased from Harlan Inc. (Madison, WI). Mice were maintained on a 12-h light/dark cycle and allowed feed and water ad libitum. Each treatment group contained at least three animals. Mice were injected i.p. with 400 mg/kg (2681 μmol/kg) Met dissolved in saline (15 μl/g) or saline alone. This dose was chosen based on the sensitivity of the analytical methods (see below) and an expected recovery of MetO in mouse urine similar to that seen for *S*-allyl cysteine sulfoxide in rats given *S*-allyl cysteine (Krause et al., 2002). Mice were sacrificed after 15, 30, or 60 min using a CO_2 chamber. Liver (0.2 g) and blood samples were obtained and immediately placed on ice. Plasma was separated from blood samples by centrifugation at 4°C in an Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany) at 5000*g* for 10 min. Plasma was then removed and placed into a separate vial. In some cases, plasma was combined from two mice to obtain a 300-μl sample. Ice-cold 0.9% NaCl (3:1 vol) was added to liver samples which were then immediately homogenized using an OMNI 2000 homogenizer (Omni International, Waterbury, CT). Liver and plasma samples were then deproteinated with ice-cold ethanol (3:1) and centrifuged at 5000*g* for 10 min in a Beckman J2-21M centrifuge (Beckman Coulter, Inc., Fullerton, CA). The supernatant was removed and taken to dryness as described above. The residue was redissolved in 475 μl of H_2O acidified to pH 2.4 with 1 M H_2SO_4. Samples were then filtered with an Acrodisc LC-13 membrane filter (Pall Gelman Sciences, Ann Arbor, MI) before fractionation by HPLC (described below).

For urinary analysis of metabolites, female mouse urine was collected from 0 to 24 h pre- and postdose. Urine was pooled from two mice housed in a metabolic cage (Nalgene, Rochester, NY). Urine samples (1 ml) were then deproteinized with 50 μl of 6 M H_2SO_4 and placed on ice for 10 min. Samples were then centrifuged at 3000 rpm for 10 min. The supernatant was removed and placed into a separate vial. Water (500 μl) was then added to each sample. The supernatant was further purified using a Waters Oasis HLB 1cc (30 mg) extraction column (Waters, Milford, MA). The column was activated by washing with 1 ml of 100% methanol and 1 ml of 0.33 M H_2SO_4; 0.5 ml of sample was then loaded. Met, MetO, and NAMO were not retained and eluted immediately. NAM was eluted with 0.5 ml of 30% methanol. The process was repeated twice more (0.5-ml sample each time) using new columns to purify the remaining sample (1 ml). NAM-containing eluents were combined separately from the Met-, MetO-, and NAMO-containing eluents. All eluents were then dried as described above.

Semipreparative HPLC Fraction Collection of Metabolites. An HPLC fraction-collecting method was developed to simultaneously separate nine metabolites of interest with the exception of 3-MTPO and Met, which coeluted (Figs. 4 and 5). Two liver samples from each mouse were processed. One liver sample was used to fractionate MetO, Met, NAMO, SAH, 3-MTP, and MTA, and the second liver sample was used to fractionate SAM, 3-MTPO, and 3-MTP. All metabolite fractions in plasma were collected from one plasma sample. Urinary NAM samples were fraction-collected separately from urinary Met, MetO, and NAMO samples. All urine samples were salted out with 1 mg of K_2SO_4 before fraction collecting. HPLC analyses were carried out using a Gilson dual pump gradient-controlled system (Gilson, Inc., Middleton, WI) fitted with a semipreparative Beckman ODS 5-μm reverse-phase C_{18} column (10 × 250 mm). UV detection was used at 220 nm on a Beckman 166 detector. Injection volume was 475 μl carried out by a Gilson 234 autosampler. The mobile phase on pump A was 100% H_2O, pH adjusted to 2.5 with TFA, and pump B contained 75% methanol, pH adjusted to 2.5 with TFA. The flow rate was 3 ml/min. Metabolites were eluted using a gradient method with an initial concentration of 0% B for 8 min. It was then increased to 50% B over 10 min where it was held for 4 min. The gradient was decreased to 0% B over 4 min and was held for a total run time of 33 min. Eluent was collected from 5.0 to 5.5 min (MetO), 5.6 to 6.3 min (SAM), 6.4 to 8 min (Met, 3-MTPO), 8.1 to 9.2 min (NAMO), 15.7 to 16.9 min (SAH), 17.2 to 18.1 min (3-MTP), 18.3 to 19.4 min (NAM), and 19.6 to 20.6 min (MTA) using a Foxy Jr. fraction collector (Isco Inc., Lincoln, NE). SAH and MTA fractions were combined before the analytical HPLC analysis of these metabolites was carried out (see below). All fractions were then taken to dryness as described above except for Met- and

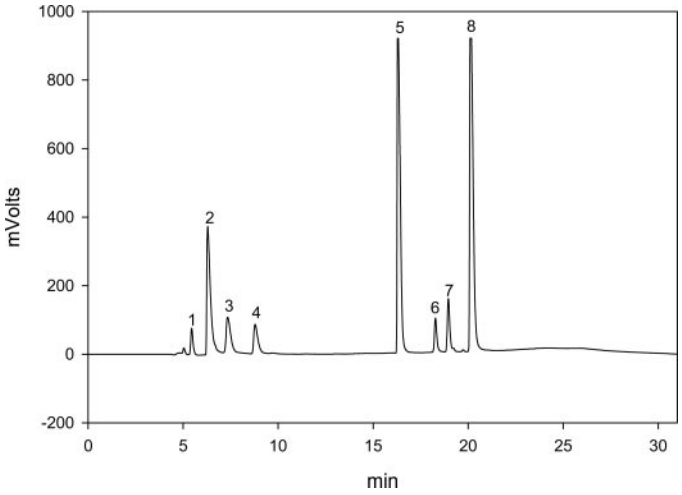

FIG. 4. Representative chromatograph showing the chromatographic separation of a mixture of synthetic standards of the nine Met metabolites of interest (200 nmol of each metabolite). 1, MetO; 2, SAM; 3, Met and 3-MTPO; 4, NAMO; 5, SAH; 6, 3-MTP; 7, NAM; 8, MTA.

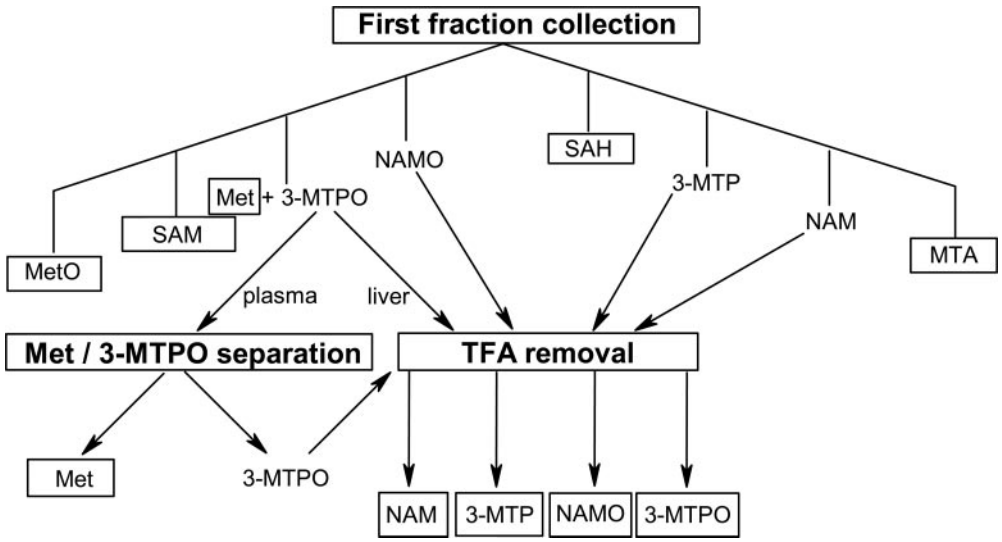

FIG. 5. Diagram of the semipreparative HPLC fraction-collecting steps for Met and nine of its metabolites of interest. Boxed metabolites in the diagram indicate the completion of fraction-collecting steps for that metabolite.

MetO-containing fractions which were first adjusted to a pH of 6.5 with 0.1 M potassium hydroxide to allow for more efficient derivatization after drying.

Separation of 3-MTPO and Met in Plasma. A fraction collection method was developed to separate Met from 3-MTPO in plasma. Dried Met/3-MTPO fractions were redissolved in 475 μl of H_2O and fraction-collected by HPLC as described above. The mobile phase was 0.1% TFA, pH adjusted to 6.5 with 4 M NaOH. Metabolites were eluted using an isocratic gradient. Eluent was collected from 4.8 to 5.6 min (3-MTPO), and 5.8 to 6.8 min (Met). The fractions were then dried as described above.

Removal of TFA via HPLC Fraction Collection. Additional fraction-collecting methods were developed for NAM, NAMO, 3-MTP, and 3-MTPO to remove residual TFA present from the mobile phase of the previous fraction collection step, because it was found to react with PBP, the derivatizing reagent used to detect these compounds. Dried liver and plasma NAM or 3-MTP fractions were redissolved in 475 μl of H_2O acidified to pH 2.4 with 1 M H_2SO_4. Dried NAMO and 3-MTPO fractions were redissolved in 235 μl of acidified H_2O and combined. NAM and 3-MTP samples were fraction-collected as described above using an isocratic gradient of 30% methanol, pH adjusted to 2.5 with 1 M H_2SO_4. Eluents were collected from 5.6 to 6.5 min (NAM) or 6.4 to 7.4 min (3-MTP). The combined NAMO and 3-MTPO sample was fraction-collected using an isocratic gradient of 100% H_2O, pH adjusted to 2.5 with 1 M H_2SO_4. Eluents were collected from 6.5 to 7.7 min (3-MTPO) and 7.9 to 9.3 min (NAMO). NAM, NAMO, and 3-MTPO fractions were then adjusted to pH 5.0 with 0.1 M KOH. 3-MTP fractions were adjusted to pH 7. All fractions were then taken to dryness as described above.

HPLC Analysis of NAM and 3-MTP. Using the above designed fraction-collecting method with a semipreparative column, linear standard curves for NAM and 3-MTP were generated in liver and plasma. The limits of quantitation for plasma NAM and 3-MTP were 8 nmol/ml. Liver limits of quantitation were 25 and 12.5 nmol/g liver, respectively. Attempts to improve the sensitivity of these methods using PBP derivatization were not successful for liver and plasma (limits of quantitation >100 nmol for both metabolites). Urinary NAM was analyzed after PBP derivatization as described below. The limit of quantitation for urinary NAM was 13 nmol/ml urine.

HPLC Analysis of Met, Met-d-O, and Met-l-O. Fractions containing Met and MetO were first derivatized with Marfey's reagent by a method adapted from Marfey (1984) to increase the molar absorptivity of Met and MetO and to resolve Met-d-O from Met-l-O (Fig. 6). In brief, dried Met- or MetO-containing fractions were redissolved in 50 μl of H_2O. Met samples from Met-dosed mice were redissolved in 200 or 400 μl of H_2O to dilute Met concentrations into the range of the Met standard curve. *S*-Methyl-L-cysteine (5 μl of a 0.6 mg/ml solution prepared in water) was then added to 25 μl of sample as internal standard. To this was added 50 μl of a 1% solution of Marfey's reagent dissolved in acetone, and 10 μl of 1 M sodium bicarbonate were then added. The reaction mixture was heated at 40°C for 1 h. After heating, 2 M HCl (5 μl) was added. The derivatized products were analyzed by HPLC with UV detection at 360 nm using a Beckman ODS 5-μm analytical column (4.6 × 250 mm), a Brownlee NewGuard guard column, a Gilson 234 autoinjector, and a Gilson 117 UV detector. Injection volume was 20 μl. The mobile phase on pump A was 50 mM triethylamine phosphate in water, and pump B contained 99% ACN. The flow rate was 1 ml/min. Metabolites were eluted using a gradient method with an initial concentration of 15% B for 15 min. It was then increased to 22% B over 15 min and then increased to 40% B over 3 min and held for 7 min. The gradient was then decreased to 15% B over 3 min and was held for 4 min for a total run time of 47 min. Retention times for derivatized Met-d-O, Met-l-O, *S*-methyl cysteine, and Met were 20.3, 21.7, 34.0, and 35.7 min, respectively. The identity of the two derivatized MetO diastereomers was determined by comparing the results of the in vivo experiments with the results obtained when Marfey's reagent was used to derivatize in vitro microsomal incubation samples previously shown to preferentially contain Met-d-O (Duescher et al., 1994). Standard curves for Met-d-O, Met-l-O, and Met were generated in liver and plasma. The limit of quantitation in plasma was 8 nmol/ml for all three metabolites. Limits of quantitation in liver were 12.5 nmol/g liver for Met-d-O and Met-l-O and 25 nmol/g liver for Met. The limit of quantitation in urine was 8 nmol/ml for all three metabolites.

HPLC Analysis of NAMO and 3-MTPO. To increase the molar absorptivity of NAMO and 3-MTPO, fractions were first derivatized with PBP by a method adapted from Durst et al. (1975). In brief, dried NAMO- or 3-MTPO-

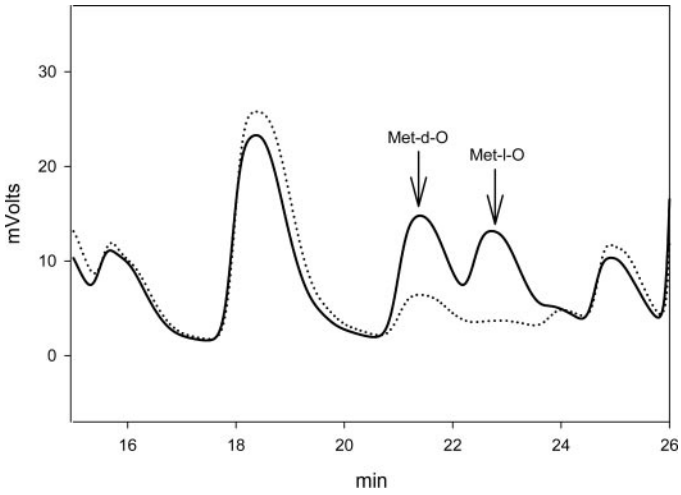

FIG. 6. Overlaid chromatographs showing the separation of Met-d-O and Met-l-O in a liver sample after derivatization with Marfey's reagent. The dashed line represents a control liver sample. The black line represents the same liver sample spiked with 25 nmol of Met-dl-O before the derivatization step.

containing fractions were redissolved in 100 μl of ACN. The samples were then sonicated thoroughly to ensure complete dissolution of NAMO or 3-MTPO. *N*-Acetyl-L-leucine (10 μl of a 0.3 mg/ml solution prepared in ACN) was then added to each sample as internal standard. Potassium carbonate (1 mg) was added to each sample, followed by sonication for 15 min. An aliquot (75 μl) of each sample was then placed in a vial to which was added 12 μl of PBP. The reaction mixture was heated at 80°C for 30 min. The derivatized products were then analyzed by HPLC with UV detection at 254 nm as described above. The mobile phase on pump A was 1% ACN, pH adjusted to 4 with TFA, whereas pump B contained 75% ACN, pH adjusted to 4 with TFA.

NAMO was eluted using a gradient method with an initial concentration of 30% B for 6 min. It was then increased to 75% B over 8 min and held for 3 min. The gradient was then decreased to 30% B over 5 min and was held for 3 min for a total run time of 25 min. Retention times for derivatized NAMO and internal standard were 7.7 and 14.3 min, respectively. Standard curves for NAMO were generated in liver and plasma, and the limits of quantitation were 50 nmol/g (liver) and 33 nmol/ml (plasma). The limit of quantitation for urinary NAMO was 12 nmol/ml.

3-MTPO was eluted using a gradient method with an initial concentration of 55% B for 9 min. It was then increased to 80% B over 5 min and held for 3 min. The gradient was then decreased to 55% B over 5 min and was held for 3 min for a total run time of 25 min. Retention times for derivatized 3-MTPO and internal standard were 5.2 and 11.7 min, respectively. Standard curves for 3-MTPO were generated in liver and plasma, and the limits of quantitation were 50 nmol/g liver and 33 nmol/ml, respectively.

HPLC Analysis of SAM, SAH, and MTA. Dried SAM and SAH/MTA fractions were analyzed by a method adapted from Wang et al. (2001). In brief, SAM- or SAH/MTA-containing fractions were redissolved in 50 μl of 0.4 M perchloric acid. Some SAM samples from Met-dosed mice were brought up in 100 or 200 μl of 0.4 M perchloric acid to dilute SAM concentrations into the range of the SAM standard curve. SAM- and SAH/MTA-containing fractions were then analyzed by HPLC with UV detection at 254 nm as described above, except that a Beckman 166 detector was used. SAM was eluted using an isocratic gradient of 50 mM sodium phosphate, pH adjusted to 3 with TFA. The retention time for SAM was 4.1 min. For SAH/MTA-containing fractions, pump A was 0.1% TFA, pH adjusted to 3 with TFA. Pump B contained 100% methanol. SAH and MTA were eluted using a gradient method with an initial concentration of 5% B for 5 min. It was then increased to 60% B over 5 min and held for 2 min. The gradient was then decreased to 5% B over 3 min and was held for 3 min for a total run time of 18 min. The retention times for SAH and MTA were 9.7 and 12.9, respectively. Standard curves for SAM, SAH, and MTA were generated. The limits of quantitation in liver were 40, 25, and 12.5 nmol/g, respectively. The limit of quantitation in plasma was 4 nmol/ml for all three compounds.

Statistics. Statistical analyses were carried out using the SigmaStat software

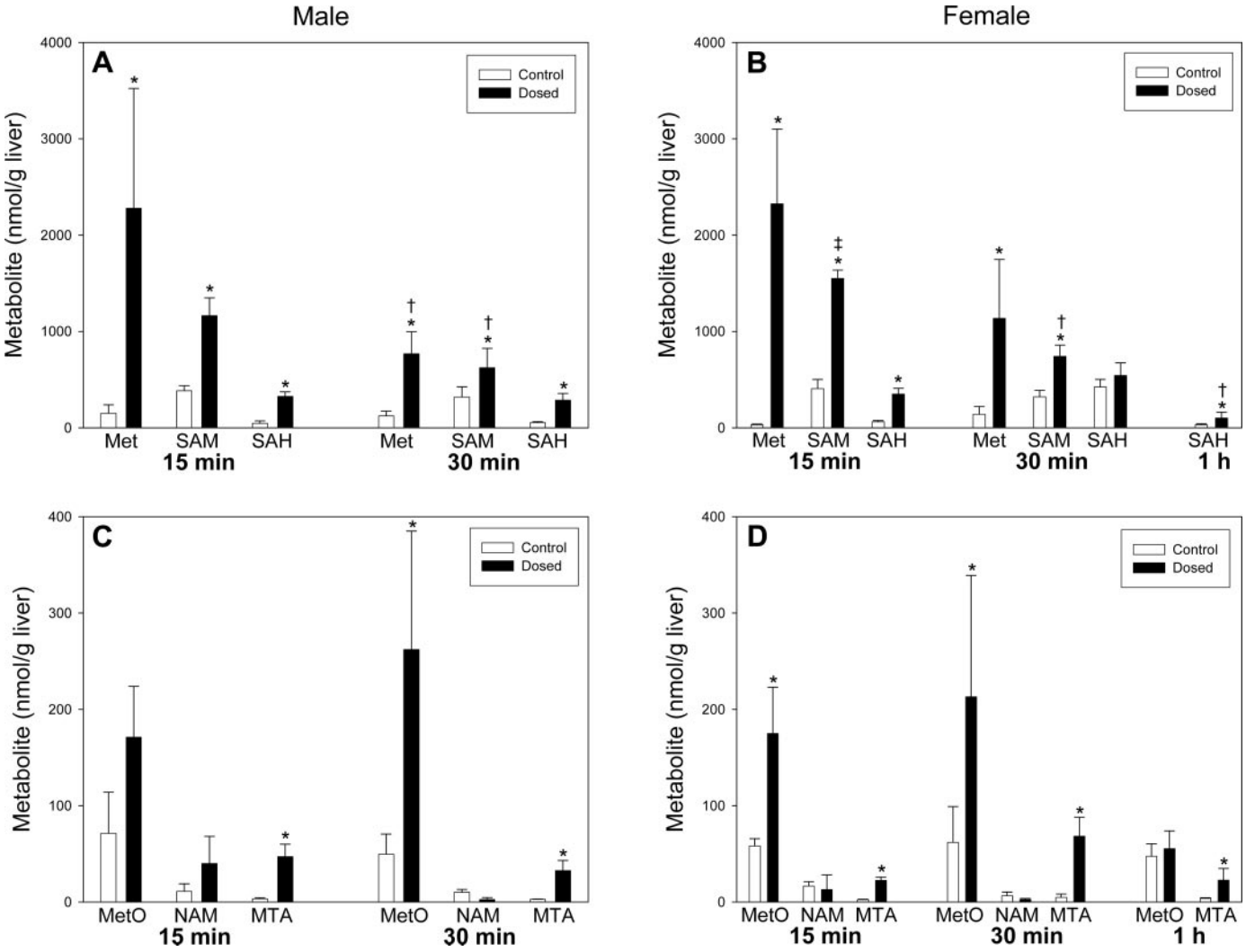

FIG. 7. Quantitation of liver Met and Met metabolites in male (A and C) and female (B and D) mice dosed with Met or saline at 15 min, 30 min, or 1 h postdose. * indicates levels that were significantly higher than the corresponding control values obtained with the saline-dosed mice. † indicates levels that were significantly lower than the corresponding values obtained from the previous time point. ‡ indicates levels that were significantly higher than the corresponding values obtained with the opposite sex at the same time point.

program (SPSS Inc., Chicago, IL). Comparisons of means were done by Student's t test. Significance level was set at $p \leq 0.05$.

Results

HPLC methods involving fraction collection and derivatization steps were developed to quantitate Met and nine of its known or potential metabolites in male and female mouse liver and plasma, 15, 30, or 60 min after a dose of Met (400 mg/kg) or saline (control). Urinary levels of Met, Met-d-O, Met-l-O, NAM, and NAMO were also determined in female mouse urine collected from 0 to 24 h postdose. The SAM, SAH, MTA, NAM, and 3-MTP methods used direct detection. Met, Met-d-O, and Met-l-O, due to their low molar absorptivities and the presence of many interfering peaks, were derivatized with Marfey's reagent before analysis. NAMO and 3-MTPO were analyzed after derivatization with PBP. All metabolite peaks were identified based on their coelution with reference compounds. Increases in Met, Met-d-O, SAM, SAH, MTA, and NAM levels were observed in the mouse liver and/or plasma samples, whereas 3-MTP, 3-MTPO, and NAMO were not detected.

Liver Met levels rose to similar levels at 15 min in Met-dosed animals of both sexes and were an average of 15-fold higher in males and 74-fold higher in females compared with saline-dosed mice (Fig. 7). Plasma Met levels at 15 min were 1.9 ± 0.4 mM (mean ± S.D.) and 1.7 ± 0.2 mM in Met-dosed males and females, respectively. Met liver levels fell in both males and females by 30 min, although the change was not statistically significant in males ($p = 0.054$). Plasma Met levels at 15 min were not significantly different from those observed at 30 min in either sex (1.5 ± 0.2 mM in males and 1.4 ± 0.1 mM in females).

Levels of the Met transmethylation metabolite SAM were higher than any other metabolite monitored in liver in Met-dosed mice at 15 min. SAM levels increased an average of 3-fold over controls in the liver of Met-dosed male mice and 4-fold in Met-dosed female mice by 15 min; SAM levels in Met-dosed females were significantly higher than the corresponding levels in Met-dosed males. Liver SAM levels decreased significantly by 30 min in both sexes to nearly half the levels observed at 15 min. Despite increased liver levels of SAM in dosed mice, there was no significant difference in plasma SAM levels between dosed mice and controls at either determined time point. Hepatic and plasma SAM levels in either sex at 1 h were not determined.

Liver SAH levels in Met-dosed mice of both sexes were increased 5- to 10-fold at 15 min, and this increase was still detectable at 30 min in males. Saline-dosed female mice at 30 min had high SAH levels compared with saline-dosed male mice, and these levels were similar to those of Met-dosed females at 30 min. Because of the high SAH levels in liver of saline-treated female mice at 30 min, female liver SAH levels were also determined at 1 h postdose. The levels were significantly increased over controls but had decreased compared with levels at 15 and 30 min postdose. Significant increases in SAH were also detected in the plasma of dosed mice at 15 and 30 min postdose in both sexes, with plasma SAH levels becoming trace levels at 1 h in females (Fig. 8).

Liver MTA levels rose 8-fold in Met-dosed males and females by 15 min. MTA levels were still significantly increased over controls at 30 min and 1 h, but decreased by the later time point. Increases in plasma MTA were not detected in either sex.

Increases in liver NAM were not detected in either sex at 15 or 30 min post-treatment; however, plasma NAM levels increased nearly 4-fold in Met-dosed female mice at 15 min. Increases in plasma NAM were not detected at 30 min.

The increases in liver and plasma MetO levels at 15 min were gender- and tissue-dependent, whereas both sexes and tissues exhibited significant increases in MetO levels at 30 min. Interestingly, significantly higher amounts of Met-d-O compared with Met-l-O were detected in Met- and saline-dosed males and females at all time points (Table 1). Indeed, Met-dosed males showed a significantly higher percentage of the liver and plasma total MetO at 15 min as Met-d-O compared with Met-dosed females.

Additional studies indicated that less than 0.1% of the Met dose was excreted in female mouse urine within 24 h (data not shown). Only trace amounts of urinary Met and Met-d-O, but not Met-l-O, NAM, or NAMO were recovered from the Met dose. Urinary levels of the Met transmethylation (SAM, SAH, MTA) and transamination (3-MTP, 3-MTPO) metabolites were not determined.

Discussion

Recovery of less than 0.1% of the Met dose (400 mg/kg) in the urine of female mice as Met and Met-d-O suggested that this high Met

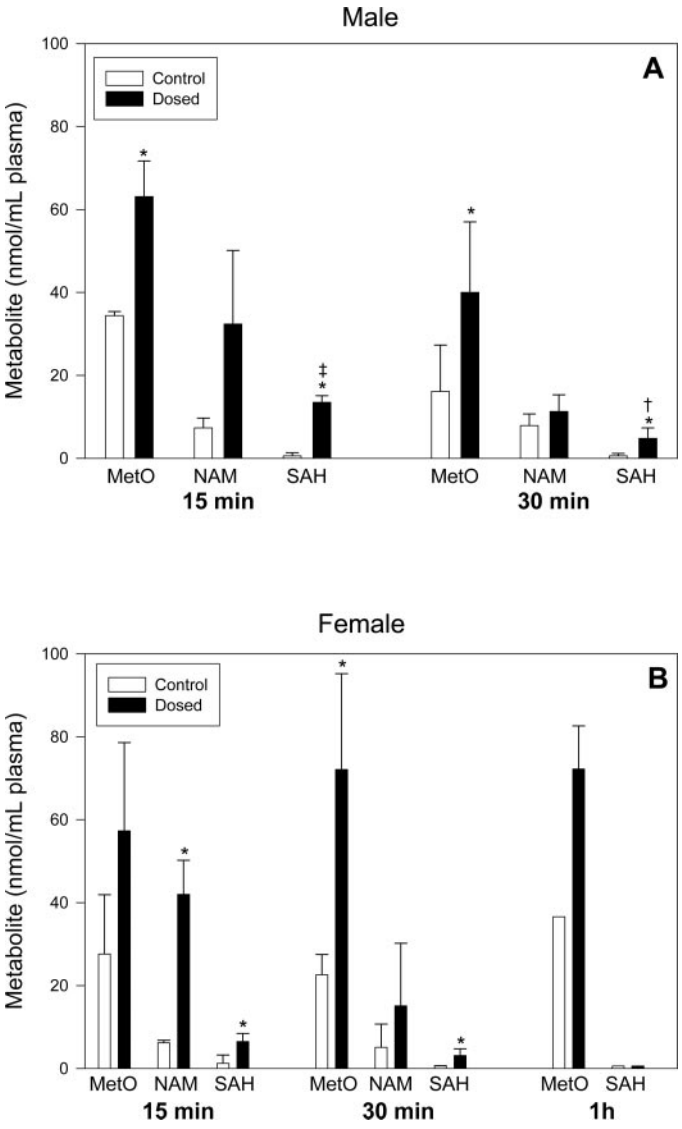

FIG. 8. Quantitation of plasma Met metabolites in male (A) and female (B) mice dosed with Met or saline at 15 min, 30 min, or 1 h postdose. * indicates levels that were significantly higher than the corresponding control values obtained with the saline-dosed mice. † indicates levels that were significantly lower than the corresponding values obtained from the previous time point. ‡ indicates levels that were significantly higher than the corresponding values obtained with the opposite sex at the same time point.

TABLE 1

Percentage of total MetO present as Met-d-O in liver and plasma of mice dosed with Met or saline at 15 or 30 min

Treatment	Liver		Plasma	
	15 min	30 min	15 min	30 min
Dosed				
Male	96 ± 4[a]	100 ± 0	98 ± 1	100 ± 0
Female	82 ± 9[b]	79 ± 25	76 ± 14[b]	87 ± 17
Control				
Male	100 ± 0	87 ± 11	99 ± 1	100 ± 0
Female	87 ± 17	85 ± 26	76 ± 23	100 ± 2

[a] Data are expressed as mean percentage ± S.D. (n = 3–5 except for 30-min dosed female mice, where n = 9).

[b] Levels were significantly lower than the corresponding values obtained with the opposite sex at the same time point.

dose was efficiently used rather than excreted into the urine. That no urinary Met-l-O was detected in the urine is consistent with the liver and plasma results where, primarily, Met-d-O and not Met-l-O was detected. NAM was not recovered in urine despite its detection in plasma at 15 min, suggesting that NAM was also further used rather than excreted.

At 15 min postdose in liver and plasma in both sexes, approximately 12% of the Met dose was recovered as Met and its metabolites. This is consistent with previous studies indicating that injected Met rapidly accumulates in many organs and tissues including the liver and plasma (Deloar et al., 1997). In the present study, half of this recovered dose was detected as Met metabolites, conversion to SAM being the primary route for Met metabolism of the pathways examined. Met incorporation into protein is also an important metabolic pathway for Met but was not monitored in the present study (Finkelstein, 1990; Deloar et al., 1997). Previously, mice lacking methionine adenosyltransferase 1A (MAT1A), the enzyme that catalyzes the conversion of Met to SAM in the liver, were shown to have 8-fold higher endogenous liver Met levels relative to control mice (Lu et al., 2001). Large increases in liver SAM levels after a dose of Met have also been reported in guinea pigs and rats (Shinozuka et al., 1971; Regina et al., 1993). Collectively, these results provide strong evidence for the importance of the transmethylation pathway in Met metabolism in mammals.

The Met transamination metabolites 3-MTP and 3-MTPO were not detected in any of the mouse tissues examined. 3-MTP formation has been detected in rat and monkey tissue homogenates incubated with Met (Steele and Benevenga, 1978; Scislowski and Pickard, 1993). 3-MTP has also been detected in the urine of humans with cystathionine β-synthase deficiency or partial hepatic MAT1A deficiency (Gahl et al., 1988; Tangerman et al., 2000). Further metabolism of 3-MTP to yield methanethiol has been demonstrated in rats (Finkelstein and Benevenga, 1986; Scislowski and Pickard, 1993). Oxidative decarboxylation of KMTB to yield 3-MTPO via 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) has been demonstrated in vitro (Crouch et al., 1997). Thus, whereas transamination of Met to KMTB may have occurred, further oxidative decarboxylation of KMTB to 3-MTP or 3-MTPO did not occur to a detectable level in the Met-dosed mice.

NAM, a novel in vivo Met metabolite, was detected in the plasma of Met-dosed female mice at 15 min whereas, of the four male Met-dosed mice at 15 min, two showed increases in NAM that were similar to those of Met-dosed females and two showed no increase in NAM. Increases in NAM were not detected at 30 min in either sex, suggesting that NAM was rapidly excreted or further metabolized. Daabees et al. (1984) reported similar Met levels in pigs dosed with Met or NAM, consistent with efficient conversion of NAM to Met.

Although NAMO has been previously identified in the urine of rats dosed with Met-dl-O (Smith, 1972), NAMO was not detected in the mouse tissues from the present study. The latter results are consistent with previous findings that rates of NAM oxidation in rabbit liver microsomes were much slower than those of Met (Elfarra and Krause, 2005).

MetO levels in Met-dosed mice of both sexes showed similar 2- to 4-fold increases at 15 and 30 min, indicating that FMO3 *S*-oxidation of Met was not the major cause of MetO formation since FMO3 is not expressed in the liver of male mice (Falls et al., 1995; Ripp et al., 1999b). The highest plasma Met concentration detected in this study (1.9 mM) is also below the K_m of FMO3 *S*-oxidation of Met (3.7–6.5 mM). Although FMO3 may have contributed to Met oxidation in female mouse liver, the enzyme responsible for Met oxidation in male mouse liver and the basis for the high levels of Met-d-O detected in both male and female mouse liver and plasma are presently unclear. Ripp et al. (1999b) detected stereoselective formation of Met-d-O in male mouse liver microsomes incubated with Met despite the absence of immunologically detectable FMO3. However, female mouse liver microsomes showed nearly 3-fold higher Met *S*-oxidase activity than males whereas, in this study, MetO formation in vivo was similar in both sexes. MetO was also detected in the blood, plasma, and urine of children with homocysteinuria (Perry et al., 1967), but the stereochemistry of the detected MetO was not investigated. Interestingly, Gahl et al. (1988) detected exclusively Met-d-O in the urine of a human with MAT1A deficiency that caused a 20- to 30-fold elevation of plasma Met (0.72 mM).

Hernandez et al. (2004) recently identified a novel FMO gene cluster in mice and humans. This gene cluster consisted of several FMO pseudogenes in humans, but in mice, three of these genes were predicted to be functional. The expression levels and catalytic properties of these FMOs are not yet known, but their potential involvement in Met *S*-oxidation cannot be ruled out at this time. Our laboratory previously characterized a high-affinity Met *S*-oxidase activity with some FMO characteristics in human and rabbit liver microsomes with apparent K_m values of 70 and 40 μM, respectively (Ripp et al., 1999a). The activity was inhibited greater than 50% by methimazole and was shown not to be mediated by cytochrome P450s or reactive oxygen species. However, this *S*-oxidase activity was less diastereoselective than that seen in the present study, producing 50 to 70% of the total MetO formed as Met-d-O. Furthermore, this activity was not detected in male or female mouse liver microsomes. Thus, differences exist in the properties of the in vivo Met *S*-oxidase activity detected in this study and the high-affinity Met *S*-oxidase activity previously characterized in vitro.

Nonenzymatic oxidation of Met by oxygen species may have contributed to the in vivo Met *S*-oxidase activity (Schoneich, 2005). Furthermore, multiple MetO reductases capable of reducing either MetO diastereomer (Sharov et al., 1999; Moskovitz et al., 2002; Vougier et al., 2003) may also have affected the stereochemical composition of MetO detected in mouse tissues. Support for the latter hypothesis is provided by the finding that racemic MetO was as effective in nutrition in rats as Met (Iwami et al., 1992).

In summary, the results presented in this article describe the levels of Met and its major metabolites in mice after a 400 mg/kg i.p. dose of Met. Because male and female mice exhibited similar MetO levels and the percentage of the total MetO present as Met-d-O inversely correlates with FMO3 expression levels in these tissues, a major role for FMO3 in MetO formation in mice is not certain. Thus, the mechanisms involved in the formation and use of MetO, and their relative roles in overall Met metabolism in male and female mice, warrant further investigations.

References

Blom HJ, Boers G, Trijbels J, van Roessel J, and Tangerman A (1989) Cystathionine-synthase-deficient patients do not use the transamination pathway of methionine to reduce hypermethionemia and homocystinemia. *Metabolism* **38:**577–582.

Crouch NP, Adlingon RM, Baldwin JE, Lee MH, and MacKinnon CH (1997) A mechanistic rationalization for the substrate specificity of recombinant mammalian 4-hydroxyphenylpyruvate dioxygenase (4-HPPD). *Tetrahedron Lett* **53:**6993–7010.

Daabees TT, Anderson DW, Zike WL, Filer LJ, and Stegink LD (1984) Portal and vena caval plasma methionine concentrations in young pigs administered L-methionine, N-acetyl-L-methionine and N-acetyl-D-methionine. *J Nutr* **114:**1541–1547.

Deloar HM, Fujiwara T, Nakamura T, Itoh M, Imai D, Miyake M, and Watanuki S (1997) Estimation of internal absorbed dose of L-[methyl-^{11}C]methionine using whole-body positron emission tomography. *Eur J Nucl Med* **25:**629–633.

Dever JT and Elfarra AA (2006) Evidence for the involvement of flavin-containing monooxygenase 3 in the in vivo S-oxidation of L-methionine in female mice. *FASEB J* **20:**A263.

Doi JT, Goodrow MH, and Musker WK (1986) Neighboring group participation in organic redox reactions: 11. Anchimeric assistance by the carboxylate anion in aqueous iodine oxidations of 3-(alkylthio)propanoates. *J Org Chem* **51:**1026–1029.

Duescher RJ, Lawton MP, Philpot RM, and Elfarra AA (1994) Flavin-containing monooxygenase (FMO)-dependent metabolism of methionine and evidence for FMO3 being the major FMO involved in methionine sulfoxidation in rabbit liver and kidney microsomes. *J Biol Chem* **269:**17525–17530.

Durst HD, Milano M, Kikta EJ Jr, Connelly SA, and Grushka E (1975) Phenacyl esters of fatty acids via crown ether catalysts for enhanced ultraviolet detection in liquid chromatography. *Anal Chem* **47:**1797–1801.

Elfarra AA and Krause RJ (2005) Potential roles of flavin-containing monooxygenases in sulfoxidation reactions of L-methionine, N-acetyl-L-methionine and peptides containing L-methionine. *Biochim Biophys Acta* **1703:**183–189.

Falls JG, Blake BL, Cao Y, Levi PE, and Hodgson E (1995) Gender differences in hepatic expression of flavin-containing monooxygenase isoforms (FMO1, FMO3, and FMO5) in mice. *J Biochem Toxicol* **10:**171–177.

Finkelstein A and Benevenga NJ (1986) The effect of methanethiol and methionine toxicity on the activities of cytochrome c oxidase and enzymes involved in protection from peroxidative damage. *J Nutr* **116:**204–215.

Finkelstein JD (1990) Methionine metabolism in mammals. *J Nutr Biochem* **1:**228–237.

Finkelstein JD (2003) Methionine metabolism in liver diseases. *Am J Clin Nutr* **77:**1094–1095.

Gahl WA, Bernardini I, Finkelstein JD, Tangerman A, Martin JJ, Blom HJ, Mullen KD, and Mudd SH (1988) Transsulfuration in an adult with hepatic methionine adenosyltransferase deficiency. *J Clin Investig* **81:**390–397.

Halsted CH, Villanueva JA, Devlin AM, Niemela O, Parkkila S, Garrow TA, Wallock LM, Shigenaga MK, Melnyk S, and James SJ (2002) Folate deficiency disturbs hepatic methionine metabolism and promotes liver injury in the ethanol-fed micropig. *Proc Natl Acad Sci USA* **99:**10072–10077.

Hernandez D, Janmohamed A, Chandan P, Phillips IR, and Shephard EA (2004) Organization and evolution of the flavin-containing monooxygenase genes of human and mouse: identification of novel gene and pseudogene clusters. *Pharmacogenetics* **14:**117–130.

Iwami K, Higashiyama Y, and Ibuki F (1992) Reevaluation of L-methionine sulfoxide bioavailability and a difference between its epimers in normal young rats. *Nutr Res* **12:**1155–1166.

Koukouritaki SB, Simpson P, Yeung CK, Rettie AE, and Hines RN (2002) Human hepatic flavin-containing monooxygenases 1 (FMO1) and 3 (FMO3) developmental expression. *Pediatr Res* **51:**236–243.

Krause RJ, Glocke SC, and Elfarra AA (2002) Sulfoxides as urinary metabolites of S-allyl-L-cysteine in rats: evidence for involvement of flavin-containing monooxygenases. *Drug Metab Dispos* **30:**1137–1142.

Krause RJ, Ripp SL, Sausen PJ, Overby LH, Philpot RM, and Elfarra AA (1996) Characterization of the methionine-S-oxidase activity of rat liver and kidney microsomes: immunochemical and kinetic evidence for FMO3 being the major catalyst. *Arch Biochem Biophys* **333:**109–116.

Lu SC, Alvarez L, Huang ZZ, Chen L, An W, Corrales FJ, Avila MA, Kanel G, and Mato JM (2001) Methionine adenosyltransferase 1A knockout mice are predisposed to liver injury and exhibit increased expression of genes involved in proliferation. *Proc Natl Acad Sci USA* **98:**5560–5565.

Marfey P (1984) Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. *Carlsberg Res Commun* **49:**591–596.

Mashima R, Nakanishi-Ueda T, and Yamamoto Y (2003) Simultaneous determination of methionine sulfoxide and methionine in blood plasma using gas chromatography-mass spectrometry. *Anal Biochem* **313:**28–33.

Moskovitz J, Singh VK, Requena J, Wilkinson BJ, Jayaswal RK, and Stadtman ER (2002) Purification and characterization of methionine sulfoxide reductases from mouse and staphylococcus aureus and their substrate stereospecificity. *Biochem Biophys Res Commun* **290:**62–65.

Moss LR, Haynes AL, Pastuszyn A, and Glew RH (1999) Methionine infusion reproduces liver injury of parenteral nutrition cholestasis. *Pediatr Res* **45:**664–668.

Perry TL, Hansen S, and MacDougall L (1967) Sulfur-containing amino acids in plasma and urine of homocystinurics. *Clin Chim Acta* **15:**409–420.

Regina M, Korhonen V, Smith T, Alakuijala L, and Eloranta T (1993) Methionine toxicity in the rat in relation to hepatic accumulation of S-adenosylmethionine: prevention by dietary stimulation of the hepatic transsulfuration pathway. *Arch Biochem Biophys* **300:**598–607.

Ripp SL, Itagaki K, Philpot RM, and Elfarra AA (1999a) Methionine S-oxidation in human and rabbit liver microsomes: evidence for a high-affinity methionine S-oxidase activity that is distinct from flavin-containing monooxygenase 3. *Arch Biochem Biophys* **367:**322–332.

Ripp SL, Kiyoshi I, Philpot RM, and Elfarra AA (1999b) Species and sex differences in expression of flavin-containing monooxygenase form 3 in liver and kidney microsomes. *Drug Metab Dispos* **27:**46–52.

Ripp SL, Overby LH, Philpot RM, and Elfarra AA (1997) Oxidation of cysteine *S*-conjugates by rabbit liver microsomes and cDNA-expressed flavin-containing monooxygenases: studies with *S*-(1,2-dichlorovinyl)-L-cysteine, *S*-(1,2,2-trichlorovinyl)-L-cysteine, *S*-allyl-L-cysteine and *S*-benzyl-L-cysteine. *Mol Pharmacol* **51:**507–515.

Schoneich C (2005) Methionine oxidation by reactive oxygen species: reaction mechanisms and relevance to Alzheimer's disease. *Biochim Biophys Acta* **1703:**111–119.

Scislowski P and Pickard K (1993) Methionine transamination—metabolic function and subcellular compartmentation. *Mol Cell Biochem* **129:**39–45.

Sharov VS, Ferrington DA, Sqier TC, and Schoneich C (1999) Diastereoselective reduction of protein-bound methionine sulfoxide by methionine sulfoxide reductase. *FEBS Lett* **455:**247–250.

Shinozuka H, Estes LW, and Farber E (1971) Studies on acute methionine toxicity. I. Nucleolar disaggregation in guinea pig hepatic cells with methionine or ethionine and its reversal with adenine. *Am J Pathol* **64:**241–249.

Smith RC (1972) Acetylation of methionine sulfoxide and methionine sulfone by the rat. *Biochim Biophys Acta* **261:**304–309.

Steele RD and Benevenga NJ (1978) Identification of 3-methylthiopropionic acid as an intermediate in mammalian methionine metabolism in vitro. *J Biol Chem* **253:**7844–7850.

Tangerman A, Wilcken B, Levy HL, Boers GHJ, and Mudd SH (2000) Methionine transamination in patients with homocystinuria due to cystathionine β-synthase deficiency. *Metabolism* **49:**1071–1077.

Vougier S, Mary J, and Friguet B (2003) Subcellular localization of methionine sulphoxide reductase A (MsrA): evidence for mitochondrial and cytosolic isoforms in rat liver cells. *J Biochem* **373:**531–537.

Wang W, Kramer PM, Yang S, Pereira MA, and Tao L (2001) Reversed-phase high performance liquid chromatography procedure for the simultaneous determination of S-adenosyl-L-methionine and S-adenosyl-L-homocysteine in mouse liver and the effect of methionine on their concentrations. *J Chromatogr B* **762:**59–65.

Zhang R, Ma J, Xia M, Zhu H, and Ling W (2004) Mild hyperhomocysteinemia induced by feeding rats diets rich in methionine or deficient in folate promotes early atherosclerotic inflammatory processes. *J Nutr* **134:**825–830.

Ziegler DM (2002) An overview of the mechanism, substrate specificities, and structure of FMOs. *Drug Metab Dispos* **34:**503–511.

Address correspondence to: Dr. Adnan Elfarra, University of Wisconsin, School of Veterinary Medicine, 2015 Linden Drive, Madison, WI 53706-1102. E-mail: elfarra@svm.vetmed.wisc.edu