Conversion of Suspected Food Carcinogen 5-Hydroxymethylfurfural by Sulfotransferases and Aldehyde Dehydrogenases in Postmitochondrial Tissue Preparations of Humans, Mice, and Rats

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ABSTRACT

The food contaminant 5-hydroxymethylfurfural (HMF) is formed by heat- and acid-catalyzed reactions from carbohydrates. More than 80% of HMF is metabolized by oxidation of the aldehyde group in mice and rats. Sulfo conjugation yields mutagenic 5-sulfoxymethylfurfural, the probable cause for the neoplastic effects observed in HMF-treated rodents. Considerable metabolic differences between species hinder assessing the tumorigenic risk associated with human dietary HMF uptake. Here, we assayed HMF turnover catalyzed by sulfotransferases or by aldehyde dehydrogenases (ALDHs) in postmitochondrial preparations from liver, kidney, colon, and lung of humans, mice, and rats. The tissues-specific clearance capacities of HMF sulfo conjugation (CLSC) and ALDH-catalyzed oxidation (CLOX) were concentrated to the liver. The hepatic clearance CLSC in mice (males: 487 ml/min/kg bw, females: 2520 ml/min/kg bw) and rats (males: 430 ml/min/kg bw, females: 198 ml/min/kg bw) were considerably higher than those in humans (males: 21.2 ml/min/kg bw, females: 32.2 ml/min/kg bw). The ALDH-related clearance rates CLOX in mice (males: 3400 ml/min/kg bw, females: 1410 ml/min/kg bw) were higher than those of humans (males: 436 ml/min/kg bw, females: 646 ml/min/kg bw) and rats (males: 627 ml/min/kg bw, females: 679 ml/min/kg bw). The ratio of CLOX to CLSC was lowest in female mice. This finding indicated that HMF sulfo conjugation was most substantial in the liver of female mice, a target tissue for HMF-induced neoplastic effects, and that humans may be less sensitive regarding HMF sulfo conjugation compared with the rodent models.

Key words: 5-hydroxymethylfurfural; metabolism; sulfotransferase; aldehyde dehydrogenase

5-Hydroxymethylfurfural (HMF) is formed by heat- and acid-induced dehydration of hexoses. It is present in a variety of foodstuffs resulting in an average daily uptake of 4–30 mg HMF per person (Abraham et al., 2011; Husoy et al., 2008; Murkovic and Fichtler, 2006). Recently, HMF attracted considerable interest as a sustainable building block for the synthesis of polymers, such as furanic polyesters, polyamides, and polyurethanes and as alternative transportation fuel (Binder and Raines, 2009; Bredikhin et al., 2013; Buntara et al., 2011). Many efforts were directed towards the preparation of HMF from the catalytic turnover of C6-carbohydrates in biomass as a renewable source (Choudhary et al., 2013; Pagan-Torres et al., 2012; Roman-Leshkov et al., 2006). Also, the
pharmacological properties of HMF were studied. It was found to increase the oxygen affinity of human hemoglobin and to reduce sickling of erythrocytes in patients with sickle cell disease (Abdulmalik et al., 2005). A phase I clinical trial initiated by AesRx (Newton, Massachusetts) was completed evaluating the effect of single HMF doses against sickle cell anemia (ClinicalTrials.gov identifier: NCT01597401). Health concerns have to be taken into consideration when HMF is to be used as pharmacon or as a large-scale chemical building block. HMF induced neoplasia in a series of animal experiments (Suzh et al., 1994; Svendsen et al., 2009; Zhang et al., 1993). For example, it caused liver adenoma in female mice in a 2-years bioassay conducted by the National Toxicology Program (2008). The relevance of this finding for humans has been questioned because liver adenomas were also detectable in control animals and a dose-response relationship was not observed (Abraham et al., 2011). Studies conducted in various standard test systems, eg, a bacterial reverse mutation test using Salmonella typhimurium strains (Lee et al., 1995a), an HPRT test and an in vitro comet assay in V79 cells (Janzowski et al., 2000), a sister chromatid exchange assay in V79 cells (Glatt et al., 2005), and the micronucleus test in peripheral blood cells of mice (National Toxicology Program, 2008), showed that HMF is barely genotoxic. However, 5-sulfoxyethylfurfural (SMF) formed from sulfo conjugation of HMF was mutagenic (Lee et al., 1995b; Suzh et al., 1994). Also, HMF was mutagenic in V79 cells expressing human (h) sulfotransferase (SULT) 1A1, which was accompanied by formation of specific DNA adducts N6-(2-formylfuran-5-y1)methyl-2′-deoxyadenosine (N6-FMF-da) and N6-(2-formylfuran-5-y1)methyl-2′-deoxyguanosine (N6-FMFD0) (Monien et al., 2012). Thus, it is possible that neoplastic effects of HMF originate from SULT-catalyzed bioactivation.

The extent of DNA damage caused by HMF exposure in a particular tissue depends partially on the expression of certain SULT forms and their specific catalytic efficiencies for HMF turnover. Recent studies of HMF sulfo conjugation by individual SULT forms showed that primarily SULT1A1 orthologues from humans, mice, and rats contribute to HMF bioactivation (Sachse et al., 2014). However, due to the lack of consistent data about SULT expression in all 3 species the reported enzyme activities do not allow a comparative quantitative assessment of HMF sulfo conjugation. Also, the internal dose of SMF may be influenced by various other species- and tissue-dependent factors, such as blood perfusion and detoxification of HMF and SMF. For an improved understanding of the hazard potential related to human HMF exposure, we studied HMF sulfo conjugation and oxidative turnover to 5-hydroxymethyl-2-furoic acid (HMFA), the most important pathway of HMF metabolism (Germond et al., 1987), in liver, kidney, lung, and colon of humans, mice, and rats. Michaelis-Menten parameters KM and VMAX of HMF turnover allowed estimating tissue-specific clearance rates of HMF sulfo conjugation and HMF oxidation in all 3 species (Obach et al., 1997).

MATERIALS AND METHODS

Chemicals. HPLC-grade methanol, 2-propanol, formic acid, and acetic acid were from Carl Roth GmbH (Karlsruhe, Germany), and HPLC-grade water was prepared using a Milli-Q Integral Water Purification System from Millipore Merck (Darmstadt, Germany). [13C6]D-glucose was purchased from Silantes GmbH (Munich, Germany). HMFA was from Toronto Research Chemicals (Toronto, Canada) and HMF and all other reagents (analytical grade) were from Sigma-Aldrich (Steinheim, Germany). SMF was synthesized as described recently (Monien et al., 2009).

Synthesis of [13C6]HMF. The [13C6]D-glucose was converted to [13C6]HMF via a biphasic reaction consisting of an aqueous phase for the Lewis acid-catalyzed dehydration and an organic layer for continuous extraction of the product (Pagan-Torres et al., 2012). A portion of 2.7 mg (20 μmol) AlCl3 was dissolved in 4 ml saturated brine and 203 mg (1091 μmol) [13C6]D-glucose was added. The solution was mixed with 8 ml 2-sec-butylpheno! and the mixture was refluxed at 160°C for 24 h. The 2-sec-butylphenol was removed and diluted with 60 ml hexane. The organic phase was extracted 3 times with 20 ml water. The water fractions were pooled and extracted 3 times with 100 ml tetrahydrofuran. The organic phase was carefully removed at 50 mbar and 50°C. Caution: [13C6]HMF may evaporate as well at lower pressure or at higher temperatures. The analysis of the [13C6]HMF by ultra performance liquid chromatography (UPLC)-UV-MS/MS showed that the yield was 43.5%. The [13C6]HMF was employed for sulfation without further purification. [13C6]NMR (600 MHz, dimethyl sulfoxide-d6) δ = 178.0 (CO, d), 161.7–162.6 (C5, t), 151.2–152.2 (C2, t), 124.2–124.9 (C3, t), 109.3–110.1 (C4, t), 55.7–56.1 (C-OH, d). The [13C]NMR spectrum is shown in Supplementary Figure S1 of the supplementary data.

Synthesis of [13C6]SMF. The sulfation of [13C6]HMF was basically performed as described (Monien et al., 2009). Briefly, 14 mg (107 μmol) of [13C6]HMF was dissolved in 2 ml dimethylformamide (DMF) under argon. The solution was cooled down to 0°C and 34 mg (214 μmol) pyridine·SO3 was added. The solution was stirred for 5 h at 0°C. Subsequently, 642 μl of 500 mM NaOMe (321 μmol) in methanol was added. After stirring for 30 min, the mixture was added dropwise to 30 ml of cold ether. The suspension was centrifuged and the precipitate was washed 2 times with 10 ml cold ether and dried for 60 min under high vacuum. [13C6]SMF was subjected to preparative HPLC using a Prep LC 150 (Waters, Eschborn, Germany) with a SunFire Prep C18 OBD column (5 μm, 19 × 150 mm; Waters) coupled to a photo diode array detector 996 (Waters). The eluents were 5% methanol in water (solvent A) and methanol (solvent B). A 20-min linear gradient was applied starting from 100% solvent A to 50% solvent A at a flow rate of 20 ml/min. In order to minimize the loss of the reactive [13C6]SMF (t1/2 = 120 min at 37°C in the presence of water) the eluents were refrigerated to 4°C and glass tubes in the fraction collector were kept on ice. The fractions containing [13C6]SMF were pooled. The purity of [13C6]SMF was >99% as indicated by UPLC-UV-MS/MS. A standard solution of 428 nM [13C6]SMF in 2-propanol was prepared and stored at −80°C.

Synthesis of [13C6]HMFA. Three milligrams (22.7 μmol) [13C6]HMFA was dissolved in 7 ml of an aqueous solution of 100 mM glucose/sodium hydroxide (pH 10.0), 20 mM β-nicotinamide adenine dinucleotide (NAD+), and 7.7 mg cytosolic protein prepared from transgenic S. typhimurium TA100 expressing human (h) aldehyde dehydrogenase (ALDH) 3A1 (Kollock et al., 2009). The reaction mixture was incubated at 37°C for 24 h, diluted with 7 ml methanol and centrifuged at 15,000 × g for 10 min. The clear supernatant was concentrated under reduced pressure and the remaining aqueous solution was subjected to purification using the preparative HPLC described above with a Delta Pak C4 column (15 μm, 19 × 300 mm; Waters). The product was eluted with water (solvent A) and methanol (solvent B) using a 20-min linear gradient starting from 100% solvent A to 60% solvent A at a flow rate of 15 ml/min. Both solvents were acidified with 0.25% acetic acid.
acid and 0.25% formic acid. After removal of the solvents under reduced pressure, $[^{13}\text{C}]$HMFA was purified again using a 20-min linear gradient starting from 100% solvent A (2% methanol) to 100% solvent B (75% methanol) at a flow rate of 15 ml/min. The product fractions were pooled and the purity (≥ 99%) was determined via UPLC and UV detection. Following dilution with methanol the concentration (1.69 µM $[^{13}\text{C}]$HMFA) was determined using a standard calibration line of commercially available HMFA, which was linear in the range between 20 nM and 50 µM.

Postmitochondrial supernatants of tissue samples. Samples of human liver, lung, kidney, and colon, 5 of each organ and each gender, were generously provided by Biopredic International (Rennes, France). The samples contained healthy tissue from the margins around sites of tumor resections, whereas colon tissue samples were mainly from patients with diverticular disease. After surgical intervention the samples were stored at −80°C. The studies were conducted by the ethic committee of the University of Potsdam (Germany). Animal tissues (liver, lung, kidney, and colon) were obtained from FVB/N mice and Wistar rats (Charles River Laboratories, Sulzfeld, Germany). Tissue samples of 0.2–1.0 g were homogenized in 3 ml KCP medium per gram of tissue (150 mM potassium chloride, 10 mM sodium phosphate buffer, pH 7.4) using a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 9000 x g for 20 min and the protein content of the supernatant (postmitochondrial supernatant [PMS]) was determined. Aliquots volumes between 100 and 500 µl were stored at −80°C.

**HMF sulfo conjugation in the presence of PMS.** Sulfo conjugation of HMF was determined in reaction mixtures of 100 µl containing 25–200 µg protein, 50 mM potassium phosphate buffer (pH 7.4), 5 mM MgCl$_2$, 50 µM 3′-phosphaadenosine-5′-phosphosulfate (PAPS) and different concentrations of HMF. Previous experiments ensured that measurements were recorded under “linear” conditions, ie, doubling of incubation time or protein concentration was doubling the amount of SMF formed. The reactions were stopped after 15 min by adding 300 µl of a solution of 428 nM $[^{13}\text{C}]$SMF in cold 2-propanol. The mixture was vortexed briefly and centrifuged at 15 000 x g for 10 min. The clear supernatants were analyzed by UPLC-MS/MS. Incubations were performed in duplicate.

Quantification of SMF by UPLC-MS/MS. SMF and $[^{13}\text{C}]$SMF were analyzed using an Acquity UPLC system (Waters) coupled to a tandem-quadrupole mass spectrometer Quattro Premier XE (Waters). Samples of 4 µl were injected onto a SunFire C18 column (5 µm, 3 x 150 mm; Waters). The mobile phase consisted of 2 solvents: 10 mM ammonium acetate/methanol (95:5, solvent A) and acetonitrile/methanol (95:5, solvent B). The gradient for the elution of SMF and subsequent reequilibration of the column was as follows: 0–1 min, 95% A; 1–3 min, 95–20% A; 3–4 min, 5% A; 4–6 min, 95% A. The flow rate was 0.5 ml/min and the column was kept at room temperature (20°C). The mass spectrometer was operated in the negative ion mode. A multiple reaction monitoring (MRM) method was devised for specific detection and quantification of SMF using the fragmentation transitions m/z = 204.9 → 95.9 and m/z = 204.9 → 80.9. The corresponding transitions were used to monitor the internal standard $[^{13}\text{C}]$SMF, m/z = 210.9 → 95.9 and m/z = 210.9 → 80.9. The tune parameters were: temperature of the electrospray source 120°C; desolvation temperature 475°C; desolvation gas: nitrogen (800 l/h); cone gas: nitrogen (100 l/h); collision gas: argon (indicated cell pressure ~5 x 10$^{-3}$ mbar). For the fragmentation of SMF ($[^{13}\text{C}]$SMF), collision energies were 18 and 23 eV for the transitions m/z = 204.9 → 80.9 (m/z = 210.9 → 80.9) and for m/z = 204.9 → 95.9 (m/z = 210.9 → 95.9), respectively. The dwell time was set to 100 ms, and capillary voltage was set to 0.35 kV. The cone and RF1 lens voltages were 32 V and 0.1 V, respectively.

HMFA oxidation in the presence of PMS. The reactions were performed in 100 µl mixtures containing 2.5–20 µg protein, 50 mM potassium phosphate buffer (pH 7.4), 5 mM NAD$, and different concentrations of HMF. Initial experiments ensured that the incubation conditions were in a linear range. After incubation for 15 min at 37°C, the reactions were stopped by adding 200 µl methanol containing 1.69 µM $[^{13}\text{C}]$HMFA. The samples were centrifuged for 10 min at 15 000 x g and the HMFA content of the supernatant was determined by UPLC-MS/MS. Incubations were performed in duplicate.

Quantification of HMFA by UPLC-MS/MS. HMFA and $[^{13}\text{C}]$HMFA were analyzed with the UPLC-MS/MS system described above. Here, an HSS T3 column (1.8 µm, 2.1 × 100 mm; Waters) was used and the analytes were eluted with a 3-min linear gradient of water (solvent A) and acetonitrile (solvent B) starting from 98% solvent A to 50% solvent A and a flow rate of 0.35 ml/min. Both solvents were acidified with 0.25% acetic acid and 0.25% formic acid. Three specific fragmentations for HMFA (m/z = 143.1 → 125.1, m/z = 143.1 → 97.1 and m/z = 143.1 → 79.1) were monitored together with the corresponding transitions of $[^{13}\text{C}]$HMFA (m/z = 149.1 → 131.1, m/z = 149.1 → 102.1 and m/z = 149.1 → 84.1) using collision energies of 10, 15, and 20 eV, respectively. The transitions of m/z = 143.1 → 79.1 and m/z = 149.1 → 84.1 were used as quantifiers whereas the presence of the other fragmentations ensured the specificity of detection. The tune parameters were as follows: temperature of the electrospray source 100°C; desolvation temperature 490°C; desolvation gas nitrogen (850 l/h); cone gas nitrogen (50 l/h); collision gas argon (indicated cell pressure ~ 5 x 10$^{-3}$ mbar). The dwell time was set to 50 ms and capillary voltage was set to 0.75 kV. The cone voltage and the RF1 lens voltage were set to 18 V and 0.1 V, respectively.

**HMF clearance by sulfo conjugation and by oxidation.** Kinetic parameters were usually derived by fitting of the HMF concentration dependent turnover rates with the conventional Michaelis-Menten model using the nonlinear regression algorithm in Sigma Plot 11 from Systat Software (Erkrath, Germany). The HMF sulfo conjugation in PMS of some tissue samples was described better under consideration of substrate inhibition (Houston and Kenworthy, 2000):

$$V = \frac{V_{\text{MAX}}}{1 + K_M/[\text{HMFA}]} + \frac{[\text{HMFA}]/K_I}{(1 + [\text{HMFA}]/K_I)}$$  \hspace{1cm} (1)

with the rate of the reaction V, the substrate concentration [HMFA], the Michaelis-Menten constant $K_M$, the maximum rate $V_{\text{MAX}}$ and the substrate inhibition constant $K_I$.

The tissue-specific clearance rates (CL) were estimated from the Michaelis-Menten parameters by scaling up (Obach et al., 1997; Soars et al., 2002):

$$\text{CL} = \frac{V_{\text{MAX}}}{K_M} \times \frac{\text{protein in PMS}}{\text{tissue}} \times \frac{\text{body weight}}{\text{body weight}}$$ \hspace{1cm} (2)

For a consistent evaluation, we used the initial slope of the HMF turnover rates determined at the 3 lowest concentrations.
as intrinsic clearance $V_{\text{MAX}}/K_{\text{M}}$. The relative tissue weight of human liver was 24.7 and 25.4 g/kg body weight for males and females, respectively, and of human lung 18.3 and 17.5 g/kg body weight for males and females, respectively. The kidney weight was 4.7 g/kg body weight for both sexes (de la Grandmaison et al., 2001). Yamanaka et al. (2007) reported 30 g/kg body weight for colon from both sexes. The tissue weights of FVB/N mice and of Wistar rats were determined when the organs were removed. The data for the calculation of the clearance rates are summarized in Supplementary Tables S3 and S4 (mice) and Supplementary Tables S5 and S6 (rats) of the supplementary data.

RESULTS

Quantification of SMF by Isotope-Dilution UPLC-MS/MS

In previous studies, we used UPLC-MS/MS and external calibration lines of SMF to assess HMF sulfo conjugation by different SULT forms in vitro (Sachse et al., 2014) and in FVB/N mice in vivo (Monien et al., 2009). To reduce the technical inaccuracy we synthesized [13C6]SMF as an internal standard for the quantification of the reaction product. Analogous to the unlabeled substance, the daughter scan of [13C6]SMF showed 2 collision-induced fragmentations: the dissociation of the sulfate ion radical ($m/z = 210.9 \rightarrow 95.9$) and of the protonated sulfonate ion ($m/z = 210.9 \rightarrow 80.9$) (Figure 1). Based on these transitions an MRM method for the specific detection of [13C6]SMF and the simultaneous recording of SMF ($m/z = 204.9 \rightarrow 95.9$ and $m/z = 204.9 \rightarrow 80.9$) was developed.

The solution of [13C6]SMF in 2-propanol was added to terminate the incubation reactions and the clear supernatants were directly injected for MRM analysis. Figure 2 shows the chromatography of SMF and [13C6]SMF in a sample containing 100 μg mouse kidney protein and 10 mM HMF. The background of the chromatograms was essentially free of interfering signals. The limit of detection (LOD) for the quantification of SMF was estimated from the background integral of the "quantifier" trace (the total ion current of both transitions) at the precise retention time in incubations without HMF (‘controls’). The LOD was defined as

![FIG. 1. Fragmentation pattern of [13C6]SMF observed by negative ESI-MS/MS collision-induced dissociation. The asterisks denote the presence of 13C. Principal fragment ions of [13C6]SMF (parent ion with $m/z = 210.9$) were the sulfonate ion, HSO$_3^-$ ($m/z = 80.9$) and the sulfate ion radical, SO$_4^{2-}$ ($m/z = 95.9$).](image1)

![FIG. 2. LC-MS/MS chromatograms of an incubation with mouse kidney protein (1 mg/ml of PMS) and HMF (10 mM) fortified with the sulfo group donor PAPS (50 μM). After stopping the incubation, fragmentations of SMF, $m/z = 204.9 \rightarrow 95.9$ and $m/z = 204.9 \rightarrow 80.9$ (upper panels), were monitored together with the transitions $m/z = 210.9 \rightarrow 95.9$ and $m/z = 210.9 \rightarrow 80.9$ (lower panels) of the isotope-labeled standard [13C6]SMF (1.28 pmol/injection). The ratio of the sums of both peak areas was used to calculate the SMF content in the incubation. Control samples without HMF did not show a signal at the retention time of SMF.](image2)
of [13C6]HMFA using the analogous transitions (m/z 143.1, 149.1, 131.1, 125.1, m/z 102.1 and m/z 97.1). The simultaneous detection of the internal reference compound [13C6]HMFA allowed the HMFA content in the incubation mixtures. The mass spectrometric detector scan of [13C6]HMFA is shown in Supplementary Figure S2 of the supplementary data. Exemplary chromatograms for the detection of HMFA in the data are depicted in Supplementary Figure S3 of the supplementary data. The LOD was calculated from the arithmetic mean area of the background noise at the precise retention time of HMFA from 10 incubations without HMF plus 3 SD. The resulting LOD was 50 nM HMFA regardless of the tissue, which corresponds to 200 fmol HMFA per injection.

HMF Oxidation in PMS From Tissue Homogenates

The oxidation rates were determined at concentrations between 1 and 1250 μM HMF in the presence of PMS samples from humans, mice and rats (Figure 4). An inhibitory effect was not detected up to 1250 μM HMF. Most of the data sets were described well with the standard Michaelis-Menten equation. The coefficients of determination (R²) reflected the goodness of the fittings. The data resulting from incubations of particular tissue PMS were not approximated adequately with the Michaelis-Menten equation, i.e., the HMF turnover with cytoplasmic protein from human kidney (R² = 0.701–0.851), human liver (R² = 0.826–0.929; compare Figure 4A) and human lung (R² = 0.791–0.970). Also, with hepatic PMS of female mice the fitting curve deviated to some extent from the data points (R² = 0.924–0.979, compare Figure 4B). In these cases, fittings with a Michaelis-Menten model including 2 components, V = (V MAX,1 + [HMF]/(K M,1 [HMF])) + (V MAX,2 + [HMF]/(K M,2 [HMF])), yielded good approximations (data not shown). The K M,1 values of the high binding affinity components were in the order of magnitude of the apparent K M values derived from the approximations with the standard Michaelis-Menten equation. The low binding affinity component (K M,2, V MAX,2) described the slow increase in the turnover rate becoming apparent in the range of > 100 μM HMF (Figure 4A). Due to the limitations of our data sets with 1250 μM as the highest HMF concentration it was not possible to derive K M,2 values with sufficient confidence. To ensure a consistent evaluation of all data sets, we used the standard Michaelis-Menten model. The resulting values for K M and V MAX are summarized in Table 2.

It was evident that the K M values of ALDH-catalyzed HMF oxidation in human tissues were usually lower than those of the other species. A sexual dimorphism was observed in mice. The apparent K M of hepatic HMF oxidation in male animals (9.4 μM) was about 7-fold lower compared with that in females (72.5 μM).

25 mM HMF, which was observed for most of the tissue samples included in this study. Assuming a substrate inhibitory effect, apparent K M and V MAX values (Table 1) were derived by approximations of the kinetic data with the extended Michaelis-Menten equation (equation 1). Values of R² > 0.98 (> 0.90) showed the goodness of 77% (98 %) of the 120 fittings (10 data sets of 4 tissues from three species) with the Michaelis-Menten model.

The K M and V MAX values for HMF sulfo conjugation in hepatic PMS preparations from human males (20.6 mM and 262 pmol/mg/min) were similar to those from females (21.8 mM and 317 pmol/mg/min) (Figure 3A and Table 1). In contrast, we observed pronounced sexual dimorphisms in mice and rats (Figs. 3B and C and Table 1). The K M in female liver of mice (1.6 mM) was about 2-fold lower compared with that in male liver (3.0 mM), while the V MAX was about 2-fold higher. In rats, the K M values of hepatic HMF sulfo conjugation in male and female rats were similar, but the V MAX was more than 2-fold higher in male liver (766 pmol/mg/min) compared with that in female liver (308 pmol/mg/min).

Quantification of HMFA by Isotope-Dilution UPLC-MS/MS

Previous studies showed that 80%–90% of a dose of [13C6]HMF was metabolized via oxidation of the aldehyde group in rats and mice (Godfrey et al., 1999). In order to compare the ALDH-catalyzed turnover of HMF to HMFA in different tissues we devised an in vitro assay in which PMS samples were incubated with HMF and NAD⁺. The formation of HMFA was monitored by the characteristic mass spectrometric fragmentations (m/z 143.1, 149.1, 131.1, 125.1, m/z 102.1 and m/z 97.1). The simultaneous detection of the internal reference compound [13C6]HMFA using the analogous transitions (m/z 143.1–131.1, m/z 149.1–102.1 and m/z 149.1–94.1) allowed quantifying the HMFA content in the incubation mixtures. The mass spectrometric detector scan of [13C6]HMFA is shown in Supplementary Figure S2 of the supplementary data. Exemplary chromatograms for the detection of HMFA and [13C6]HMFA are depicted in Supplementary Figure S3 of the supplementary data. The LOD was calculated from the arithmetic mean area of the background noise at the precise retention time of HMFA from 10 incubations without HMF plus 3 SD. The resulting LOD was 50 nM HMFA regardless of the tissue, which corresponds to 200 fmol HMFA per injection.

FIG. 3. HMF sulfo conjugation in PMS preparations of liver tissues from human (A), mouse (B), and rat (C). The rates at single HMF concentrations are means ± SE of 5 different samples of males (solid squares) and females (open circles). Fitting of the data with the Michaelis-Menten model including a substrate inhibitory effect at elevated HMF concentrations yielded values for the catalytic parameters K M and V MAX, which are summarized in Table 1.

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In contrast, the HMF oxidation in male human liver ($K_M = 10.3 \mu M$ and $V_{MAX} = 2370 \text{ pmol/mg/min}$) was similar to that in females ($K_M = 8.4 \mu M$ and $V_{MAX} = 2420 \text{ pmol/mg/min}$), and also there was no detectable difference of HMF oxidation in the presence of hepatic protein from male and female rats (Figure 4C).

**Clearance of HMF by Sulfo Conjugation and Aldehyde Oxidation**

The intrinsic clearance $V_{MAX}/K_M$ was used to estimate the capacities of SULT- and ALDH-catalyzed HMF turnover ($CL_{SUL}$ and $CL_{ALD}$) in each tissue according to equation 2. The clearance rates for HMF sulfo conjugation ($CL_{SC}$) are summarized in Table 1. In all three species the liver showed the highest HMF sulfo conjugation capacity. For example, in human males the hepatic clearance was about 20-fold and 6-fold greater compared to that in male and female humans, respectively. A species comparison suggested that humans and rats may be similar regarding ALDH-catalyzed HMF clearance, especially in the liver (Table 2). The capacity of hepatic HMF oxidation in mice exceeded that in the other species. In male mice, the oxidative clearance was 7.8-fold and 5.4-fold higher compared with humans and rats, respectively, and in female mice it was 2.2-fold and 2.1-fold higher compared with humans and rats, respectively.

**DISCUSSION**

The metabolic detoxification of HMF is primarily determined by the oxidation of the aldehyde moiety, which accounts for > 80% of the overall HMF turnover. The main metabolites observed in urine of rats or mice within 24 h after administration of $^{14}$C-HMF were HMF A (77.5%–84.9%), N-[5-hydroxymethyl-2-furyl]glycine (1.3%–7.9%), and 2-furan dicarbonylic acid (2.0%–5.9%) (Godfrey et al., 1999). Also in humans exposed to high amounts of HMF by consumption of dried plum juice or dried plums all of the urinary metabolites detected were either HMF or conjugates thereof (Prior et al., 2006). Sulfo conjugation contributes less to HMF metabolism. When HMF was intravenously injected in male mice, only about 500 ppm of the dose was converted into SMF reaching the plasma (Monien et al., 2009). Comparable data on HMF sulfo conjugation are not available for rats, humans, and female mice. For the assessment of the human hazard potential associated with the internal SMF carcinogenicity studies. To this end, we determined clearance of HMF sulfo conjugation ($CL_{SC}$) and oxidation ($CL_{OX}$) in 4 different tissues from humans, mice, and rats, ie, liver (target tissue of HMF-induced neoplasia in the mouse [National

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**TABLE 1. Kinetic Parameters ($K_M$ and $V_{MAX}$) and Clearance Rates ($CL_{SC}$) of HMF Sulfo Conjugation in Different Tissues of Human, Mouse, and Rat**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Species</th>
<th>$K_M$ (mM)</th>
<th>$V_{MAX}$ (pmol/mg/min)</th>
<th>$CL_{SC}$ (μl/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Human</td>
<td>20.6 ± 1.7</td>
<td>262 ± 69</td>
<td>21.2 ± 6.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.7 ± 3.4</td>
<td>15.1 ± 3.0</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25.5 ± 3.9</td>
<td>37.7 ± 10.2</td>
<td>0.60 ± 0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37.5 ± 9.6</td>
<td>134 ± 53</td>
<td>1.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>3.0 ± 0.1</td>
<td>360 ± 23</td>
<td>487 ± 21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.8 ± 1.0</td>
<td>540 ± 21</td>
<td>48.8 ± 3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.6 ± 1.3</td>
<td>9.9 ± 1.7</td>
<td>0.80 ± 0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.7 ± 0.6</td>
<td>290 ± 24</td>
<td>7.7 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>5.3 ± 0.5</td>
<td>766 ± 61</td>
<td>430 ± 34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.8 ± 0.2</td>
<td>28.8 ± 7.0</td>
<td>3.2 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.4 ± 5.6</td>
<td>2.1 ± 0.5</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27.6 ± 2.3</td>
<td>45.7 ± 8.4</td>
<td>0.32 ± 0.06</td>
</tr>
</tbody>
</table>

---

Values of apparent $K_M$ and $V_{MAX}$ are means ± SE of measurements from 5 tissue samples. The HMF sulfo conjugation rates were fitted with the Michaelis-Menten model including a substrate inhibitory effect observed at high HMF concentrations (compare Figure 3) as described in the method section (equation 1).

$CL_{SC}$ values (means ± SE of 5 different samples) were calculated separately for all individual PMS samples from human, mouse and rat. The parameters for the calculation according to equation 2 in the method section are summarized in Tables S1 to S6 of the supplementary data. Due to the absence of an inhibitory effect at high HMF concentrations the sulfo conjugation was best fitted with the standard Michaelis-Menten model.

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In the liver, the capacity of hepatic sulfo conjugation was 80% and 85% in male and female humans, respectively, and about 80% and 86% in male and female humans, respectively, and about 80% and 86% in male and female rats, respectively.
cases, the apparent $K_M$ values in PMS were similar to the $K_M$ values reported for the HMF turnover catalyzed by individual SULT forms highly expressed in liver. For instance, the $K_M$ of hepatic HMF sulfo conjugation in female mice (1.6 ± 0.1 mM) was in agreement with the observation that Sult1a1 (K_M = 2.0 ± 0.3 mM) is the principal SULT form in mouse liver (Alnouti and Klaassen, 2006; Honma et al., 2001). However, the HMF sulfo conjugation in a particular tissue may be catalyzed usually by various different SULT forms. In humans, for example, the K_M values of hepatic HMF sulfo conjugation, 20.7 mM in males and 18.5 mM in females, may not be explicable by hSULT1A1-mediated catalysis alone. Other important SULT forms expressed at considerable levels in human liver besides hSULT1A1 (53%, $K_M = 3.2$ mM) are hSULT2A1 (27%, $K_M > 50$ mM) and SULT1B1 (14%, $K_M = 13.3$ mM) (Riches et al., 2009; Sachse et al., 2014). Yet, the HMF turnover in most of the tissue PMS was usually well described by a standard, 1-component Michaelis-Menten equation (Figure 3). The resulting apparent $K_V$ values may be regarded as average values resulting from the contribution of different SULT forms.

The most important reaction of HMF detoxification is probably the ALDH-catalyzed turnover to HMFA (Godfrey et al., 1999). In the ALDH superfamily three classes contribute primarily to xenobiotic metabolism in mammals: ALDH1 (cytosolic), ALDH2 (mitochondrial) and ALDH3 (cytosolic). Isoenzymes of hALDH1 and hALDH2, which are expressed constitutively in the liver at high levels (Sladek, 2003; Stewart et al., 1996), have relatively low K_M values in the micromolar range for many aliphatic and aromatic aldehydes (Klyosov, 1996; Wang et al., 2009). In contrast, hALDH3 was described as a class of isoenzymes with relatively high K_M values (Yin et al., 1989). To our knowledge kinetic data about HMF oxidation by single ALDH forms were not published. Other representative substrates for small aromatic aldehydes are benzaldehyde and 2-furaldehyde, a structural congener of HMF. Freshly isolated ALDH1 from human liver oxidized benzaldehyde ($K_M = 0.25$ μM) and 2-furaldehyde ($K_M = 4.8$ μM) (Wang et al., 2009). Purified recombinant hALDH1A1 catalyzed the turnover of benzaldehyde ($K_M = 0.4$ μM) and 2-furaldehyde ($K_M = 5.8$ μM) with similar efficiencies (Solobodowska et al., 2012). In contrast, hALDH3A1 was active at much higher concentrations of benzaldehyde ($K_M = 148$ μM) or 2-furaldehyde ($K_M > 1000$ μM (Solobodowska et al., 2012); $K_M = 20$ mM (Yin et al., 1989)). The current data suggested that the liver may be the most important tissue also for HMF oxidative turnover catalyzed by ALDH and that at least 2 ALDH forms with distinct kinetic properties may contribute to hepatic HMF oxidation to HMFA in humans. The $K_M$ values resulting from the fitting of ALDH-catalyzed HMF turnover in human liver with a standard Michaelis-Menten equation (Table 2) were determined primarily by the increase in turnover rates in the range of low substrate concentrations (Figure 4). The $K_M$ values are in the same range than those of the hALDH1A1-catalyzed turnover of benzaldehyde and 2-furaldehyde (Solobodowska et al., 2012). This observation suggests that hALDH1A1 may be the high affinity component of HMF oxidation and that the low binding affinity component, which was not adequately described by our experiments, may be hALDH3A1. Initial experiments showed that mitochondria did not contribute significantly to HMF turnover (Supplementary Table S13 in the supplementary data). Especially in mice and in rats the ALDH-catalyzed HMF turnover observed in the presence of mitochondrial preparations was minute in comparison to that in the PMS samples.

Various unknown parameters hinder drawing comparative conclusions on the overall HMF sulfo conjugation in the three species. For example, the influence of SMF detoxification by glutathione conjugation, the quantitative impact of the tissue-specific

**FIG. 4.** HMF oxidative turnover to HMFA in PMS preparations of liver tissues from human (A), mouse (B), and rat (C). The rates at single HMF concentrations are means ± SE of 5 different samples of males (solid squares) and females (open circles). Applying the Michaelis-Menten equation yielded satisfactory fittings for HMF oxidation by hepatic protein samples from male mouse ($R^2 = 0.987$) and male ($R^2 = 0.986$) as well as female rats ($R^2 = 0.987$) but not for the data sets of male humans ($R^2 = 0.888$), female humans ($R^2 = 0.886$), and female mouse ($R^2 = 0.962$), which deviated from the classic Michaelis-Menten curve in the range of HMF > 100 μM. In order to ensure a consistent evaluation, we used the initial slope of all data sets as intrinsic clearance $V_{MAX}/K_M$ for the calculation of CL_{DOS}. 

Toxicology Program, 2008), kidney (SMF-induced renal toxicity in the mouse (Bauer-Marinovic et al., 2012)), lung, and colon (induction of aberrant crypt foci was reported in rats [Zhang et al., 1993] and in mice [Svendsen et al., 2009]).

In all three species most of the capacities of HMF sulfo conjugation were concentrated in the liver (Table 1). This is primarily due to high expression levels of different SULT forms (Glatt, 2002) but also due to the relative weight of the liver, which exceeds that of the other tissues (de la Grandmaison et al., 2001). The comparison of apparent $K_M$ values of hepatic HMF sulfo conjugation with the $K_M$ values of individual SULT forms (Sachse et al., 2014) indicated that HMF turnover in PMS samples may be catalyzed by several different SULT forms. In some
TABLE 2. Kinetic Parameters (KM and VMAX)² and Clearance Rates (CLOX)² of HMF Oxidation in Different Tissues of Human, Mouse, and Rat

<table>
<thead>
<tr>
<th></th>
<th>KM µM</th>
<th>VMAX pmol/mg/min</th>
<th>CLOX ml/min/kg bw</th>
<th></th>
<th>KM µM</th>
<th>VMAX pmol/mg/min</th>
<th>CLOX ml/min/kg bw</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>10.3 ± 1.1</td>
<td>2370 ± 130</td>
<td>436 ± 57</td>
<td></td>
<td>8.4 ± 0.6</td>
<td>2420 ± 230</td>
<td>646 ± 116</td>
</tr>
<tr>
<td>Kidney</td>
<td>12.0 ± 5.4</td>
<td>1420 ± 80</td>
<td>30.5 ± 6.2</td>
<td></td>
<td>9.3 ± 1.3</td>
<td>1490 ± 110</td>
<td>345 ± 3.3</td>
</tr>
<tr>
<td>Lung</td>
<td>13.0 ± 7.9</td>
<td>717 ± 129</td>
<td>36.7 ± 7.9</td>
<td></td>
<td>6.9 ± 1.0</td>
<td>464 ± 118</td>
<td>333 ± 7.4</td>
</tr>
<tr>
<td>Colon</td>
<td>8.8 ± 1.4</td>
<td>614 ± 110</td>
<td>40.8 ± 10.3</td>
<td></td>
<td>15.5 ± 8.9</td>
<td>704 ± 172</td>
<td>37.3 ± 11.1</td>
</tr>
<tr>
<td><strong>Mouse</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>9.4 ± 0.7</td>
<td>11900 ± 200</td>
<td>3400 ± 210</td>
<td></td>
<td>72.5 ± 9.8</td>
<td>9240 ± 440</td>
<td>1410 ± 100</td>
</tr>
<tr>
<td>Kidney</td>
<td>1960 ± 240</td>
<td>7100 ± 960</td>
<td>4.2 ± 0.7</td>
<td></td>
<td>1490 ± 130</td>
<td>8660 ± 1390</td>
<td>19.9 ± 4.5</td>
</tr>
<tr>
<td>Lung</td>
<td>31.2 ± 3.4</td>
<td>662 ± 150</td>
<td>11.6 ± 1.9</td>
<td></td>
<td>14.7 ± 2.0</td>
<td>366 ± 64</td>
<td>16.0 ± 2.8</td>
</tr>
<tr>
<td>Colon</td>
<td>96.4 ± 8.6</td>
<td>3420 ± 270</td>
<td>8.8 ± 0.6</td>
<td></td>
<td>107 ± 15</td>
<td>3970 ± 180</td>
<td>13.3 ± 1.7</td>
</tr>
<tr>
<td><strong>Rat</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>87.7 ± 24.4</td>
<td>9900 ± 390</td>
<td>627 ± 112</td>
<td></td>
<td>100 ± 37</td>
<td>9360 ± 510</td>
<td>679 ± 127</td>
</tr>
<tr>
<td>Kidney</td>
<td>64.6 ± 4.2</td>
<td>10600 ± 300</td>
<td>344 ± 6</td>
<td></td>
<td>105 ± 8</td>
<td>8990 ± 160</td>
<td>102 ± 1</td>
</tr>
<tr>
<td>Lung</td>
<td>51.4 ± 6.3</td>
<td>2680 ± 210</td>
<td>10.6 ± 2.0</td>
<td></td>
<td>39.7 ± 3.5</td>
<td>2900 ± 190</td>
<td>15.6 ± 1.9</td>
</tr>
<tr>
<td>Colon</td>
<td>79.5 ± 11.0</td>
<td>1330 ± 80</td>
<td>3.9 ± 0.7</td>
<td></td>
<td>77.5 ± 8.9</td>
<td>1330 ± 110</td>
<td>5.7 ± 0.4</td>
</tr>
</tbody>
</table>

a Values are means ± SE of measurements from 5 tissue samples. The HMF oxidation rates were fitted with the standard Michaelis-Menten model (compare Figure 4) as described in the method section.

b CLOX values (means ± SE of 5 different samples) were calculated separately for all individual PMS samples. The parameters for the calculation according to equation 2 in the method section are summarized in Tables S7 to S12 of the supplementary data.

directed import and export of SMF (Bakhiya et al., 2009), and important physiologic parameters, eg, blood-perfusion of single tissues, were not considered. It is also not clear whether the experimental conditions of the in vitro study were suitable for the simulation of the enzymatic turnover of HMF in vivo. However, the marked differences between HMF sulfo conjugation and aldehyde oxidation detected in this study allowed for some plausible conclusions. The bioactivation and detoxification of HMF was compared by predicting values of CLSC and CLOX from the intrinsic clearance (VMAX/KM) of sulfo conjugation and aldehyde oxidation in PMS samples prepared from human, mouse, and rat tissues (Obach et al., 1997). Generally, the ALDH-related clearance rates CLOX were about 3 orders of magnitude greater compared with those of HMF sulfo conjugation CLSC. This is in accordance to previous reports about HMF metabolism in rodents in vivo (Godfrey et al., 1999; Monien et al., 2009). The CLOX in liver of male and female mice was about 7000- and 560-times greater, respectively, than the CLSC. The data from rats indicated an opposite sex-dependency. In male and female rats, the hepatic CLOX values exceeded those of CLSC by factors of about 1460-fold and 3400-fold, respectively. In humans, there was no sexual dimorphism regarding the HMF turnover. The ratio of CLOX to CLSC values determined in hepatic PMS samples of male and female humans were 20.600 and 20.100, respectively. The data suggested that there are prominent species differences in HMF sulfo conjugation and detoxification. The ratio of CLOX to CLSC was lowest in liver of female mice indicating a relatively high quantitative importance of HMF sulfo conjugation. This correlates with the observation that oral exposure to 188 mg HMF/kg body weight and 375 mg HMF/kg body weight for 104 weeks led to increased incidences of hepatocellular adenomas in female but not in male mice and also not in rats (National Toxicology Program, 2008). In humans, the ratio of hepatic CLOX to CLSC was about 36-times greater compared with that in female mice suggesting that ALDH-mediated HMF detoxification may be quantitatively more important in humans than in female mice. If the neoplastic effects of HMF were due to the formation of SMF, the current data would indicate that female mice may be the most sensitive organism for HMF-induced tumorigenicity when compared with rats and humans. This finding supports the notion that the carcinogenic risk resulting from human dietary exposure to HMF may be of minor importance.

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SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

REFERENCES


