The Metabolism and Toxicity of Furosemide in the Wistar Rat and CD-1 Mouse: a Chemical and Biochemical Definition of the Toxicophore

Dominic P. Williams, Daniel J. Antoine, Philip J. Butler, Russell Jones, Laura Randle, Anthony Payne, Martin Howard, Iain Gardner, Julian Blagg, and B. Kevin Park

The Drug Safety Research Group, Department of Pharmacology, University of Liverpool, Liverpool, United Kingdom (D.P.W., D.J.A., L.R., B.K.P.); Pharmacokinetics Dynamics and Metabolism (R.J., A.P., M.H., I.G.) and Sexual Health Chemistry (J.B.), Pfizer, Sandwich, United Kingdom; and Cyprotex, Nacclesfield, United Kingdom (P.J.B.)

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ABSTRACT

Furosemide, a loop diuretic, causes hepatic necrosis in mice. Previous evidence suggested hepatotoxicity arises from metabolic bioactivation to a chemically reactive metabolite that binds to hepatic proteins. To define the nature of the toxic metabolite, we examined the relationship between furosemide metabolism in CD-1 mice and Wistar rats. Furosemide (1.21 mmol/kg) was shown to cause toxicity in mice, but not rats, at 24 h, without resulting in glutathione depletion. In vivo covalent binding to hepatic protein was 6-fold higher in the mouse (1.57 ± 0.98 nmol equivalent bound/mg protein) than rat (0.26 ± 0.13 nmol equivalent bound/mg protein). In vivo covalent binding to mouse hepatic protein was reduced 14-fold by a predose of the cytochrome P450 (P450) inhibitor, 1-amino-benzotriazole (ABT; 0.11 nmol equivalent bound/mg protein, respectively). In vivo covalent binding to mouse hepatic protein was reduced 14-fold by a predose of the cytochrome P450 (P450) inhibitor, 1-amino-benzotriazole (ABT; 0.11 ± 0.04 nmol equivalent bound/mg protein), which also reduced hepatotoxicity. Administration of [14C]furosemide to bile duct-cannulated rats demonstrated turnover to glutathione conjugate (8.8 ± 2.8%), γ-ketocarboxylic acid metabolite (22.1 ± 3.3%), N-dealkylated metabolite (21.1 ± 2.9%), and furosemide glucuronide (12.8 ± 1.8%). Furosemide-glutathione conjugate was not observed in bile from mice dosed with [14C]furosemide. The novel γ-ketocarboxylic acid, identified by nuclear magnetic resonance spectroscopy, indicates bioactivation of the furan ring. Formation of γ-ketocarboxylic acid was P450-dependent. In mouse liver microsomes, a γ-ketoenal furosemide metabolite was trapped, forming an N-acetylcysteine/N-acetyl lysine furosemide adduct. Furosemide (1 mM, 6 h) became irreversibly bound to primary mouse and rat hepatocytes, 0.73 ± 0.1 and 2.44 ± 0.3 nmol equivalent bound/mg protein, respectively, which was significantly reduced in the presence of ABT, 0.11 ± 0.03 and 0.21 ± 0.1 nmol equivalent bound/mg protein, respectively. Furan rings are part of new chemical entities, and mechanisms underlying species differences in toxicity are important to understand to decrease the drug attrition rate.

Furosemide (4-chloro-N-furfuryl-5-sulfamoylanthranilic acid; FS) is a highly potent loop diuretic frequently used in the treatment of edematous states associated with cardiac, renal, and hepatic failure and for the treatment of hypertension (Ponto and Schoenwald, 1990). FS has been shown to produce massive hepatic necrosis in mice by a mechanism independent of its diuretic action (Mitchell et al., 1974, 1976). Incubation with inhibitors of P450 to a chemically reactive metabolite, which binds covalently with hepatic macromolecules both in vivo and in vitro (Mitchell et al., 1976). Incubation with inhibitors of P450 enzymes decreased the incidence and severity of FS-induced hepatic necrosis (Mitchell et al., 1974). This hepatotoxicity is reproducible in mice with at least eight other

ABBREVIATIONS: FS, furosemide; P450, cytochrome P450; NAC, N-acetyl cysteine; NAL, N-acetyl lysine; GSH, reduced glutathione; ABT, 1-aminobenzotriazole; PEG, polyethylene glycol; DMSO, dimethyl sulfoxide; ALT, alanine transaminase; HPLC, high-pressure-performance liquid chromatography; GSSG, oxidized glutathione; LC-MS, liquid chromatography-mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; MTS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; amu, atomic mass unit; SG, glutathione adduct.
furan-containing compounds including 2-acetyl furan and furan itself (Mitchell et al., 1974). Experiments using radiolabeled FS demonstrate that the entire drug molecule becomes covalently bound to protein. It was suggested that this was a consequence of metabolic activation of the furan ring, possibly through epoxidation (Mitchell et al., 1976). It has more recently been demonstrated with 4-ipomeanol that a double N-acetyl cysteine (NAC) and N-acetyl lysine (NAL) conjugate can be formed from in vitro incubations. This structure supports the formation of an 1,4-enedial metabolite of the furan ring (Baer et al., 2005). This has also been shown to be the case for teucrin A, a furan-containing diterpenoid germander extract associated with hepatotoxicity in man (Druckova and Marnett, 2006). Enhancement of FS-protein covalent binding, in the presence of an epoxide hydrolase inhibitor, has been reported (Wirth et al., 1976). However, chemical definition of the ultimate toxic metabolite of FS has not been reported.

Previous studies have demonstrated that hepatic glutathione (GSH) levels were not altered by the administration of a toxic dose of FS to mice (Mitchell et al., 1974). FS has also been shown to have no effect on the cytosolic or mitochondrial GSH content in CD-1 mice (Wong et al., 2000). Furthermore, no GSH depletion was observed in rat hepatocytes treated with FS (Takagi et al., 1991). However, 70% depletion in cellular GSH content has been reported in isolated CD-1 mouse hepatocytes (Grewal et al., 1996). The postulated mechanisms of toxicity induced by the reactive metabolite(s) of FS include binding to critical cellular macromolecules and subsequent disruption of calcium homeostasis (Mitchell et al., 1974; Massey et al., 1987).

The objective of the current study is to fully clarify the metabolism of FS in vivo in Wistar rats and CD-1 mice and to identify the nature of chemically reactive species formed and attempt to define the role these species play in initiation of hepatotoxicity. We have investigated the role of cytochrome P450 in the formation of reactive metabolite(s) of FS and made an assessment of the role of furan bioactivation in species differences in susceptibility to furosemide hepatotoxicity. In vivo biliary studies have been employed to identify cytochrome P450-dependent metabolite formation and species differences in metabolism between the Wistar rat and CD-1 mouse to define the relationship in susceptibility to FS hepatotoxicity. Rodent hepatocyte investigations have been used to demonstrate a relationship among metabolism, covalent binding, GSH depletion, and cytotoxicity in vitro. Finally, species differences in covalent binding are demonstrated in the Wistar rat, CD-1 mouse, and human to define the relative hazard of protein modification FS poses to hepatic protein of each species.

Materials and Methods

Materials. [14C]FS (specific activity, 629 MBq/mmol) and unlabeled FS were provided by Pfizer (Kent, UK) and determined to be >98% pure. The [14C] label was incorporated into the carbonyl carbon atom of FS and thus expected to be incorporated in all major metabolites. GSH, NADPH (tetratal sodium salt), 1-aminobenzotriazol (ABT), polyethylene glycol (PEG), Hanks’ balanced salt solution, pH 7.4, and dimethyl sulfoxide (DMSO) were purchased from Sigma (Poole, UK). Infinity alanine transaminase (ALT) (GPA) liquid kits were purchased from Alpha Laboratories (Eastleigh, UK). Bio-Rad Protein Assay Dye Reagent was purchased from Bio-Rad Laboratories Ltd. (Hemel Hempstead, Hertfordshire, UK). All solvents were of HPLC grade and were the products of Fischer Scientific plc (Loughborough, UK).

Experimental Animals. The protocols described were undertaken in accordance with criteria outlined in a license granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Animal Ethics Committee.

Investigation of the Hepatotoxicity of FS in the Wistar Rat and CD-1 Mouse. Male CD-1 mice (25–35 g) and male Wistar rats (200–250 g) were administered a single i.p. injection of FS (1.21 mmol/kg; n = 4 per group) in PEG. Animals treated with vehicle alone were used as controls. Either 5 or 24 h after administration, animals were killed by cervical dislocation, and blood was collected by cardiac puncture. Blood samples were stored at 4°C and allowed to clot overnight. Livers were collected, rinsed, and stored in 10% neutral buffered formalin for histological analysis and/or snap-frozen. Furthermore, a group of male CD-1 mice (25–35 g) were administered a single injection of ABT (745 μmol/kg in PEG i.p.) 1 h before the dose of FS.

Determination of the in Vivo Levels of Covalent Binding of FS to Hepatic Tissue in the Wistar Rat and CD-1 Mouse. To assess in vivo levels of hepatic covalent binding, male CD-1 mice (25–35 g) were administered FS (1.21 mmol/kg; n = 4 per group) and [14C]FS (10 μCi) i.p. An injection of ABT (745 μmol/kg in PEG i.p.) was given to an additional group of mice 1 h before the administration of [14C]FS and unlabeled FS. After 1, 5, or 24 h, animals were killed by cervical dislocation, and blood was collected as described previously. Livers were removed, rinsed, and stored at −80°C before use. To determine the levels of covalent binding, a portion (∼50 mg) of liver was taken from mice and Wistar rats that had been administered [14C]FS (10 μCi) and 1.21 mmol/kg FS. This was homogenized and subjected to an exhaustive solvent extraction procedure as described previously (Pirmohamed et al., 1995b). The amount of radiolabeled FS bound irreversibly to hepatic protein in vivo was determined. Aliquots of the solubilized protein were taken for quantification of radioactivity by liquid scintillation counting.

Determination of Serum Alanine Transaminase Levels. Serum was separated by centrifugation (4000 rpm) from blood that had been allowed to clot overnight at 4°C. Serum ALT levels were determined using ThermoTrace Infinity ALT Liquid stable reagent according to the manufacturer’s instructions. Hepatotoxicity was indicated by ALT levels greater than 200 U/l (Goldring et al., 2004).

Histological Examination of Murine Hepatic Sections. A transverse section of the left hepatic lobe was collected from formalin-fixed 24-h livers. Paraffin-embedded tissue sections were prepared at a thickness of 4 μm and were stained with hematoxylin and eosin for evaluation. All histological examinations were performed by a single pathologist (blinded to drug treatment) evaluating one liver section per animal, using a DMLB microscope (Leica Microsystems (UK) Ltd., Milton Keynes, UK). Hepatic sections were scored according to predefined criteria. In brief, grade 0 represented normal histology, and grade 4 represented massive hepatocellular necrosis involving entire lobules.

Determination of Glutathione Levels in Vivo in Wistar Rats and CD-1 Mice. Total hepatic glutathione (GSH + GSSG) levels were determined spectrophotometrically using a microtitrator plate assay (Vandeputte et al., 1994) based upon the 5,5'-dithiobis(2-nitrobenzoic acid)-glutathione disulfate recycling procedure first reported by Owens and Belcher (1965).

Investigation of the Biliary Metabolites of FS in the Wistar Rat and CD-1 Mouse. Adult male Wistar rats (200–400 g) and male CD-1 mice (30–35 g) were terminally anesthetized with urethane (1.4 g/l in isotonic saline; 1.0 ml/kg i.p.) and pentobarbital sodium (60 mg/kg in isotonic saline i.p.), respectively, and canulated via the trachea, jugular vein, and the common bile duct. [14C]FS (10 μCi) and 151 μmol/kg unlabeled FS dissolved in DMSO were administered i.v. over 10 min. One group of Wistar rats and one...
group of mice (n = 4 per group) were treated with ABT (745 µmol/kg i.v.) in DMSO 1 h before administration of FS. Drug-free bile was collected for 30 min before administration of FS. Bile was then collected hourly for 3 h into preweighed Eppendorfs. Protein within the bile samples was precipitated with addition of ice-cold acetonitrile. The samples were centrifuged at 2200 rpm for 10 min. The resultant supernatant was collected, transferred to fresh tubes, and evaporated to dryness under a stream of nitrogen at 37°C. The residue was stored at −20°C until analysis, when it was reconstituted in methanol (100 µl) and water (100 µl) for analysis by radio metric LC-MS.

**HPLC and LC-MS Analysis of Wistar Rat and CD-1 Mouse Bile.** The configuration of the LC-MS system and parallel radioactivity detector (Radiomatic A250; Canberra Industries, Meriden, CT) has been described previously (Madden et al., 1996). A Quattro II tandem quadrupole instrument (Micromass Ltd., Manchester, UK) fitted with the standard in-line LC-MS interface and electrospray source was employed in both positive and negative ion mode for full scanning acquisitions between m/z 100 and 1050 at 1 scan/s. The LC system consisted of two Jasco PU980 pumps (Jasco [UK], Great Dunmow, Essex, UK) and a Jasco HG-980-30 mixing module. Biliary and microsomal metabolites were resolved on an Ultracarb 5-µm C-8 column (250 × 4.6 mm) with a gradient of acetonitrile (10–60% over 30 min) in ammonium formate, pH 3.5 (10 mM) at a flow rate of 0.9 ml/min. Eluate split flow to the LC-MS interface was 40 µl/min.

**Accurate Mass Analysis of Wistar Rat Bile.** Accurate mass determinations of parent compound and metabolites were carried out on the Waters quadrupole time of flight Premier (Waters Ltd., Milford, MA) and a Jasco HG-980-30 mixing module. Biliary and microsomal metabolites were resolved on an Ultracarb 5-µm column (250 × 4.6 mm) with a gradient of acetonitrile (10–60% over 30 min) in ammonium formate, pH 3.5 (10 mM) at a flow rate of 0.9 ml/min. Eluate split flow to the LC-MS interface was 40 µl/min.

The LC-MS fraction collection system consisted of an 1100 binary pump (Agilent Tech. Inc.), 1100 diode array detector (Agilent Tech. Inc.), Accurate 51 splitter box (LC Packings, Sunnyvale, CA), LCQ Classic mass spectrometer (Thermo Electron Corp., Waltham, MA), and a 202 fraction collection (Gilion Inc., Middleton, WI). The HPLC system used a HIRB-250SP (250 × 7.75 mm) column (Hichroma, Berks, UK) with two mobile phases; water containing 0.1% formic acid (v/v) and acetonitrile containing 0.1% formic acid (v/v), at a flow rate of 2.0 ml/min. Two gradient systems were used to purify the γ-ketocarboxylic acid metabolite. Multiple injections (3 × 300 µl) of the semipurified bile extract were made, a gradient elution was performed (5% of the organic mobile phase for 1 min, increasing to 40% over 12 min), and fractions were collected. The resulting fractions were pooled, diluted 2-fold with 0.1% (v/v) formic acid in water, and reinjected (4 × 500 µl) onto the second gradient system (5% of the organic mobile phase for 1 min, increasing to 40% over 30 min). In each case, the column eluant was split with the high flow directed to the fraction collector and the low flow to the MS. The mass spectrometer was operated in negative ion mode with an electrospray ionization source. The capillary temperature was 250°C; the source voltage, 4.5 kV; the capillary voltage, −47 V; and the sheath gas flow and auxiliary gas flow were both 50 (arbitrary units). Full scan data were acquired with the fraction collector being triggered to collect when the γ-ketocarboxylic acid metabolite of FS (m/z 363) was detected. The collected fractions were combined, the solvent was removed at 30°C under a stream of nitrogen (Turbovap; Caliper Life Sciences), and the residue was stored at −20°C until analysis by NMR.

**Deuterium Exchange for Determination of γ-Ketocarboxylic Acid Formation.** Metabolite III was reconstituted in 50/50 (v/v) deuterated methanol/deuterium oxide (100% D; Euristop, Gif-sur-Yvette, France) to a final concentration of 0.25 µM before MS analysis. MS data were acquired with a Bruker 600 MHz Ultrashield Mass Selective Refractive Index detector (Bruker, Bremen, Germany) using a cryoprobe accessory (Bruker A) at 30°C utilizing proton, proton-proton (correlation spectroscopy), and proton-carbon (heteronuclear single quantum correlation and heteronuclear multiple-bond correlation spectroscopy) correlations.

**Hepatocyte Isolation from Male Wistar Rats and CD-1 Mice.** Rat and mouse hepatocytes were isolated using a modified two-step in situ collagenase perfusion based on Seglen (1976). Male Wistar rats (150–170 g) and male CD-1 mice (20–30 g) were anesthetized with sodium pentobarbital (60 mg/ml, 1 µl/g i.p.). The hepatic portal vein was cannulated, and the liver was perfused for 7 min at a constant flow rate of 12 ml/min for mice and 40 ml/min for rats, with a wash buffer containing Hanks’ balanced salt solution (from 10× stock: magnesium chloride, magnesium sulfate, sodium bicarbonate) (Invitrogen, Paisley, UK). Wash buffer was also supplemented with 5.8 mM HEPES and 4.5 mM NaHCO3. The wash step was followed by a collagenase perfusion using wash buffer supplemented with 5 mM CaCl2, 0.5 mg/ml collagenase A (Roche Applied Science, Indianapolis, IN), and 0.072 mg/ml trypsin inhibitor until the liver constituted had been digested. All wash and digestion solutions were kept at 37°C throughout the perfusion. After in situ digestion, the liver was excised, and the liver capsule was removed to release parenchymal and nonparenchymal cells. The cells were suspended in 50 ml of wash buffer supplemented with DNMase I (0.1 µg/ml) and filtered through a 125-µm mesh (Lockertex, Cheshire, UK). Cell suspensions were immediately centrifuged at 30g for 2 min, the resulting supernatant containing nonparenchymal cells was removed, and the cells were resuspended in wash buffer containing DNMase I. This centrifugation step was repeated twice at 50g with wash buffer alone; following, the cells were resuspended in an incubation buffer (wash buffer supplemented with 1 mM MgSO4, 2H2O). Hepatocyte viability was determined using a trypan blue exclusion assay using a cell counter (Casy 300, Alere, Germany).
assessed by trypan blue exclusion and only used when viability was >85%. Experimental hepatocyte incubations contained a total volume of 6 ml at a cell concentration of 2×10^6 cells/ml.

Assessment of FS-Induced Cytotoxicity in Freshly Isolated Rodent Hepatocytes. Hepatocyte incubations were carried out in an orbital shaker set at 190 rpm for 0 to 6 h with furosemide [0–2 mM in MeOH (1% v/v) and 0.1 μCi of [14C]FS in ethanol]. Some incubations were preincubated with the nonspecific P450 inhibitor ABT (5 mM, 1 h). Following incubations, cytotoxicity was determined by trypan blue exclusion (100-μl cell suspension and 20 μl of 0.4% trypan blue solution at 0, 0.5, 1, 2, 4, and 6 h). Cytotoxicity was also determined by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTS) solution using the CellTiter 96 AQ+ assay One Solution Cell Proliferation Assay kit (Promega, Madison, WI), according to the manufacturer’s instructions. Cell suspension (100 μl) was combined with MTS solution (20 μl), incubated at 37°C for 1 h, and centrifuged at 50,000 g for 2 min. The absorbance of the resulting supernatant was measured at 490 nm using a microtiter-plate reader (Dynex Technologies, Chantilly, VA).

Determination of GSH Content of Primary Rodent Hepatocytes. From the same incubations used for cytotoxicity assessment, the effect of FS on total cellular levels of GSH was determined. Cells (1×10^6) from the hepatocyte incubation were centrifuged at 50,000 g for 2 min. The resulting pellet was resuspended in 10 mM HCl. One-fifth of lysed hepatocytes was removed for protein content determination. The remaining hepatocyte lysate was combined with 6.5% (w/v) 5-sulfosalicylic acid (four parts hepatocyte lysate and one part 5-sulfosalicylic acid) and stored on ice for 10 min before centrifugation at 18,400 g for 5 min. The total GSH and GSSG content was measured at 412 nm from the resulting supernatant (Vandeputte et al., 1994). GSH content was compared with known standards ranging from 0 to 40 nmol/ml.

Investigation of FS Metabolism and Irreversible Binding in Primary Rodent Hepatocytes. The remaining portion of the hepatocyte incubation (after cytotoxicity and GSH assessment) was placed in an equal volume of ice-cold acetonitrile and centrifuged at 880g for 10 min. The resulting supernatant was evaporated under a stream of N2 in a 50°C water bath. The residue was reconstituted in 100 μl of methanol and 100 μl of water for radiochromatographic analysis. The protein pellet from the 880g spin was used to determine the levels of [14C]FS bound to the hepatocyte protein. Irreversible binding was determined by an exhaustive solvent extraction procedure using 70% methanol as described previously (Pirmohamed et al., 1995a). Aliquots of the solubilized protein (dissolved in 1 ml of 1 M NaOH at 60°C) were taken for quantification of radioactivity by liquid scintillation counting and normalized to protein concentration.

In Vitro Bioactivation of FS in CD-1 Mouse, Wistar Rat, and Human Liver Microsomes. FS (100 μM) was incubated with mouse liver microsomes (2 mg/ml), NADPH (1 mM), NAL (1 mM), and NAC (1 mM) at 37°C for 1 h. After 1 h, the reaction was terminated by the addition of 3 ml of ice-cold acetonitrile. The samples were centrifuged at 2200 rpm for 10 min. The resultant supernatant was collected, transferred to fresh tubes, and evaporated to dryness under a stream of nitrogen at 37°C. The residue was stored at −20°C until being reconstituted in methanol (100 μl) and water (100 μl) for analysis by LC-MS as described earlier. For covalent binding studies, [14C]FS (0.1 μCi, 0.6 μM) was incubated with CD-1 mouse, Wistar rat, and human liver microsomes (2 mg) in the presence and absence of NADPH (1 mM) and the presence and absence of GSH (1 mM).

Determination of Protein Concentration. Soluble protein concentration was measured using Bio-Rad protein assay reagent according to the manufacturer’s instructions.

Statistical Analysis. All results are expressed as mean ± S.E.M. Values to be compared were used for non-normality using a Shapiro-Wilk test. Student’s t tests were used when normality was indicated. A Mann-Whitney U test was used for nonparametric data. For multiple comparisons, one-way analysis of variance tests were used. All calculations were performed using Arcus Quickstat statistical software (Altrincham, UK), results were considered to be significant when p < 0.05.

**Results**

Investigation of the Hepatotoxicity of FS in the Wistar Rat and CD-1 Mouse

FS (1.21 mmol/kg i.p.) was found to be significantly toxic to mice at both 5 and 24 h (Fig. 1A), compared with control-
dosed mice. Administration of a 1-h predose of ABT (745 μmol/kg i.p.) before a toxic dose of FS provided complete protection from toxicity at 24 h. There was no increase in serum ALT levels (24 h) in mice dosed with ABT and then toxic FS compared with control-dosed mice (Fig. 1B). Administration of FS (1.21 mmol/kg i.p.) to male Wistar rats (data not shown) failed to elicit any increase in serum ALT levels at either 5 or 24 h after dosing.

Irreversible Binding of FS to Hepatic Protein in Vivo

Over a period of 24 h, the level of FS bound covalently to hepatic protein increased steadily following administration of [14C]FS (10 μCi) and 1.21 mmol/kg FS to mice (0.35 ± 0.16, 0.80 ± 0.28, and 1.57 ± 0.98 nmol equivalent bound/mg protein at 1, 5, and 24 h, respectively). The level of covalent binding was paralleled by the mean ALT activity at each time point (68 ± 28.5, 213 ± 48.8, and 1285 ± 766.9 U/l at 1, 5, and 24 h, respectively). A predose of ABT (745 μmol/kg i.p.) was shown to prevent, significantly, the binding of FS to hepatic protein at 5 and 24 h (0.09 ± 0.04 and 0.11 ± 0.04 nmol equivalent bound/mg protein). ABT also significantly reduced the level of ALT release at 5 and 24 h (160 ± 93.6 and 20.8 ± 35.5 U/l). Interestingly, a predose of ABT also dramatically reduced the amount of radiolabeled material recovered from hepatic tissue (Fig. 1C).

In Wistar rats, the level of FS bound covalently to hepatic protein was 0.26 ± 0.13 nmol equivalent bound/mg protein following a 24-h dose of [14C]FS (10 μCi) and 1.21 mmol/kg FS. Administration of ABT (745 μmol/kg i.p.) 1 h before administration of FS resulted in covalent binding levels of 0.22 ± 0.06 nmol equivalent bound/mg protein. Serum ALT levels in these rats were measured as 16.4 ± 13.5 and 4.6 ± 8.1 U/l, respectively. This data are summarized in Table 1.

Histological Examination of Murine Hepatic Sections

The histopathology data corroborated serological evidence and confirmed that FS-treated mice developed mild to moderate multifocal hepatic necrosis, predominantly within the centrilobular to midzonal regions (Fig. 2). These livers were graded using predefined criteria and had a mean histopathology score (n = 4) of 2.2. Control livers were graded 0.

Effect of FS on Hepatic GSH Levels in Vivo in Wistar Rats and Mice

FS did not have any significant effect on hepatic GSH levels in either rats or mice. Mean hepatic GSH levels were not significantly altered compared with control 1, 5, and 24 h following a 1.21 mmol/kg i.p. dose of FS to male CD-1 mice. In male Wistar rats, mean hepatic GSH levels were not significantly altered 1 h following a 1.21 mmol/kg i.p. dose of FS.

Biliary Metabolism of FS in the Wistar Rat and CD-1 Mouse

[14C]FS (10 μCi; 151 μmol/kg) administered i.v. to anesthetized male Wistar rats was rapidly excreted into bile.

![Image](https://www.jpet.aspetjournals.org/article-pdf/10.1124/jpet.117.253354/10.1124/jpet.117.253354.pdf)
Over 3 h, 21.2 ± 2.6% (mean ± S.E.M., n = 4) of the dose (37.76 μmol) was excreted into bile. Wistar rat bile (0–1-h collection) contained four prominent radiolabeled metabolites (Fig. 3A; summarized in Table 1), also detectable by MS (Fig. 3, B–F), in addition to the parent compound (34.4 ± 3.8% of excreted dose). FS (V) corresponded to the single peak in the m/z 329-ion chromatogram (Fig. 3F). MS/MS daughter ion spectra (positive ion) of FS alone showed a major fragment at m/z 81, attributable to methyl furan (data not shown). Negative ion MS/MS daughter ion spectra of FS alone showed fragments at m/z 285 (loss of 44 amu; carboxylic acid) and at m/z 205 (further loss of 80 amu; methyl furan; data not shown). The most polar metabolite (I) corresponded to the single peak in the m/z 636-ion chromatogram (Fig. 3B) and was identified as a novel FS GSH conjugate (FS-SG; 8.7 ± 2.8% of excreted dose). MS/MS daughter ion spectra (positive ion; Fig. 4A) yielded indications of GSH conjugation. A fragment at m/z 308 was attributable to GSH. A loss of 129 amu resulting in the fragments at m/z 507 and 179 is characteristic of cleavage at the γ-glutamyl-cysteinyl peptide linkage within a GSH residue (Maggs et al., 1995). The second most polar metabolite (II) corresponded to the single peak in the m/z 249-ion chromatogram (Fig. 3C). This was identified as an N-dealkylated metabolite of FS (21.1 ± 2.9% of excreted dose). MS/MS daughter ion spectra (negative ion; Fig. 4B) showed fragments at m/z 205 (loss of 44 amu; CO₂) and 78 (NSO₂), confirming the presence of these groups with the metabolite and indicating metabolic loss of the alkyl group. A second novel metabolite (III) was also identified (22.1 ± 3.3% of excreted dose; Fig. 3D) and corresponded to the single peak in the m/z 363-ion chromatogram (Fig. 5). Accurate mass analysis of the ion indicated m/z 363.0041 (Fig. 5), consistent with an elemental composition of C₁₂H₁₂N₂O₇SCl, suggesting the addition of H₂ and O₂ to furosemide. Accurate mass MS/MS daughter ion spectra (negative ion, Fig. 5) identified fragments at m/z 319.0155 (loss of 43.9871 amu; COO⁻) and m/z 275.0257 (loss of 87.9767; 2×COO⁻), respectively. The detected loss of 2×COO suggests that two carboxylic acid groups are present in the metabolite. A fragment ion at m/z 204.9828 indicated a molecular formula of C₆H₆N₂O₂SCl (±1.1-mDa error) consistent with the loss of 114.0342 (loss of methyl furan + H₂O₂) from the fragment at m/z 319. The loss of 114 amu is evidence of FS.
hydrolysis to either a dihydrodiol or a γ-ketocarboxylic acid. Further confirmation of the metabolic alteration to the furan ring was demonstrated by absence of an m/z 81 fragment, attributable to methyl furan, seen in the positive MS/MS daughter ion spectra (data not shown). However, MS/MS analysis could not differentiate between a dihydrodiol or a γ-ketocarboxylic acid metabolite of FS (see NMR analysis). Glucuronidation of FS was also evident in analysis of Wistar rat bile. FS glucuronide (IV; 12.8 ± 1.8% of excreted dose) was the least polar of the four identified metabolites and corresponded to the single peak in the m/z 505-ion chromatogram (Fig. 3E). MS/MS daughter ion spectra (negative ion; Fig. 4C) demonstrated a loss of 176 amu to provide a fragment at m/z 329. A fragment at m/z 175 was attributable to glucuronic acid with a loss of 18 amu (water), also producing a fragment at m/z 113 following further loss of 18 (water) and 44 (COO) amu. The glucuronic acid was shown not to have conjugated on the furan ring by the presence of a fragment at m/z 81, attributable to methyl furan, in positive MS/MS daughter ion spectra. A predose of ABT (745 mol/kg), 1 h before FS administration, demonstrated complete inhibition of P450-mediated metabolism of FS to the γ-ketocarboxylic acid metabolite and GSH conjugate (data not shown). Anesthetized male CD-1 mice demonstrated lower levels of biliary excretion in comparison with Wistar rats when administered i.v. with [14C]FS (10 µCi; 151 µmol/kg). Over 3 h, 11.01 ± 2.60% (mean ± S.E.M.; n = 4) of the dose (5.3 µmol) was excreted into bile. However, of this dose, 89.6 ± 24.7% was the parent compound. Radiometric HPLC demonstrated FS turnover to three minor radiolabeled metabolites with similar retention times to γ-ketocarboxylic acid (4.5 ± 1.7%), FS glucuronide (2.7 ± 1.1), and the N-dealkylated metabolite of FS (3.1 ± 1.5%) identified in Wistar rat bile. Following a predose of ABT (745 µmol/kg), 1 h before FS administration, formation of γ-ketocarboxylic acid was entirely inhibited. It is interesting to note that no FS-SG conjugate was observed to be formed in the CD-1 mouse.

Deuterium Exchange for Determination of γ-Ketocarboxylic Acid Formation

If metabolism of FS forms a γ-ketocarboxylic acid, this would contain four exchangeable protons, whereas a dihydrodiol metabolite would contain five exchangeable protons under negative ion MS. Negative ion MS of metabolite III gave pre-exchange peaks at 363.003 and 364.9962 (chlorine isotope). Postdeuterium exchange (+D2O + CD3OD), peaks were observed at 367.0222 and 369.0211, giving a strong indication that a γ-ketocarboxylic acid metabolite of FS was formed.

Nuclear Magnetic Resonance Analysis of the γ-Ketocarboxylic Acid Metabolite of FS from Wistar Rat Bile

The 1H NMR spectrum of the metabolite (Fig. 6, A and B) shows the absence of the furan ring and the presence of two mutually coupling CH2 groups. This suggests that the metabolism has affected the furan ring. The 13C NMR shifts of these two CH2 groups, obtained from a heteronuclear single quantum correlation experiment, are observed at 35.0 and 29.1 ppm, respectively. Analysis of the heteronuclear multiple-bond correlation spectroscopy data shows correlations from both of these CH2 groups to carbonyl carbons at 175.5 and 204.7 ppm. A further correlation to the carbonyl at 204.7 ppm is observed from a CH2 singlet at 4.386 ppm. These observations are consistent with metabolism of the furan ring to a C(O)CH2CH2COOH moiety (data not shown).

Metabolism and Cytotoxicity of FS toward Primary Wistar Rat and CD-1 Mouse Hepatocytes

Murine Hepatocytes. FS was metabolized to N-dealkylated FS, γ-ketocarboxylic acid, and FS glucuronide by murine
hepatocytes. In hepatocyte incubations in the presence of ABT, only the FS glucuronide metabolite could be detected (see Table 2). Similar to the in vivo situation, there was no significant dose- or time-dependent GSH depletion observed in CD-1 mouse hepatocytes incubated with FS (Table 2; Fig. 7, A and C). Significant dose- and time-dependent decreases in cell viability were observed both by trypan blue dye exclusion and the MTS assay. Cytotoxicity was correlated with dose- and time-dependent increases in irreversible binding of FS to hepatocyte protein (Fig. 7, B and D). In the presence of ABT, there was a significant decrease in irreversible binding, and cell viability returned to control values (Table 2).

**Wistar Rat Hepatocytes.** FS was metabolized to a FS-SG conjugate, N-dealkylated FS, γ-ketocarboxylic acid, and FS glucuronide by rat hepatocytes. Hepatocyte incubations in the presence of ABT, only the FS glucuronide metabolite could be detected (see Table 3). In contrast to the in vivo situation, there was significant dose- or time-dependent GSH depletion observed in Wistar rat hepatocytes incubated with FS (Table 3; Fig. 8, A and C). Significant dose- and time-dependent decreases in cell viability were observed both by trypan blue dye exclusion and the MTS assay. Cytotoxicity was correlated with dose- and time-dependent increases in irreversible binding of FS to hepatocyte protein (Fig. 8, B and D). In the presence of ABT, there was a significant decrease in irreversible binding, and cell viability returned to control values (Table 3).

**In Vitro Bioactivation of FS in CD-1 Mouse Liver Microsomes and Covalent Binding to Mouse, Rat, and Human Liver Microsomes**

Covalent binding was observed in CD-1 mouse, Wistar rat, and human microsomal preparations in the presence of NADPH. Inclusion of GSH in these incubations returned the level of irreversible binding to background level, as observed in the absence of NADPH (Fig. 9A). Coincubation of NAL and NAC with FS in a NADPH-dependent CD-1 mouse liver microsomal system led to the identification of a FS NAL/NAC double adduct. MS/MS daughter ion spectra analysis of m/z 660 (negative ion; Fig. 6A).

**TABLE 2**
Overview of in vitro metabolism and bioactivation of FS in CD-1 mouse hepatocytes

Primary CD-1 mouse hepatocytes were exposed to FS (1 mM FS for both metabolism and toxicity studies; this included 0.1 μCi/ml 14C FS for metabolism and covalent binding studies) for 6 h. Metabolism and toxicity dependence upon CYP450 was assessed through the use of ABT (5 mM, 1 h predose). Values are expressed as mean ± S.E.M. of four independent hepatocyte isolations. For the metabolic studies, values are expressed as mean percentage of total metabolites following radiochromatographic integration of peaks: *p < 0.05, FS compared with control; and †, p < 0.05 FS and ABT compared with FS alone (Mann-Whitney U test).

<table>
<thead>
<tr>
<th>Metabolism</th>
<th>FS Glutathione Conjugate</th>
<th>N-Dealkylated FS</th>
<th>γ-Ketocarboxylic Acid</th>
<th>FS Glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS</td>
<td>33.3 ± 6.0</td>
<td>11.0 ± 1.7</td>
<td>19.3 ± 3.9</td>
<td>33.3 ± 5.8</td>
</tr>
<tr>
<td>FS + ABT</td>
<td>68.2 ± 3.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>Cytotoxicity</th>
<th>MTS Reduction</th>
<th>Total GSH Content</th>
<th>Covalent Binding of FS to Hepatic Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trypan Blue Dye Exclusion</td>
<td></td>
<td></td>
<td>nmol equivalent bound/mg protein</td>
</tr>
<tr>
<td>FS</td>
<td>63.1 ± 4.3*</td>
<td>65.9 ± 4.8*</td>
<td>77.6 ± 19.6</td>
<td>0.73 ± 0.1*</td>
</tr>
<tr>
<td>FS + ABT</td>
<td>93.6 ± 3.8†</td>
<td>102.2 ± 5.2†</td>
<td>89.4 ± 10.1</td>
<td>0.11 ± 0.03†</td>
</tr>
</tbody>
</table>

![Fig. 6. A, 1H NMR spectrum of isolated γ-ketocarboxylic acid metabolite of FS in d4-MeOH. Inset, magnification of the mutually coupled protons of the methyl groups from C19 and C20. B, 1H-1H NMR spectrum (correlation spectroscopy) of isolated γ-ketocarboxylic acid metabolite of FS in d4-MeOH. The mutually coupled protons from the methyl groups (20-H2 and 19-H2) of the metabolized furan ring.](image-url)
9B) showed that the loss of 412 amu was a prominent peak in the spectra (m/z 249), indicating loss of the NAL and NAC groups, along with the pyrrole ring, effectively leaving N-dealkylated FS. The peak at m/z 487 is most probably the result of loss of the NAC group (loss of 173 amu), retaining the pyrrole structure and NAL group. The loss of 129 amu provided a fragment at m/z 532 that was attributable to cleavage adjacent to the cysteine sulfur atom. The fragment at m/z 205 indicated loss of the adduct-pyrrole ring structure as well as the loss of the carboxylic acid group.

**Discussion**

We have demonstrated that bioactivation of the furan ring represents the mechanism of metabolic activation and hepatotoxicity of FS. Characterization of three key novel metabolites from in vivo and in vitro studies, FS γ-ketocarboxylic...
acid, FS-SG conjugate, and a mixed N-acetyl lysine and N-acetyl cysteine conjugate, is primary evidence (Fig. 10). Bioactivation was dependent upon cytochrome P450 enzymes, led to hepatocellular necrosis, in the absence of glutathione depletion. Biliary, hepatocyte, and microsomal studies have provided evidence of two reactive, potentially toxic, metabolites, FS epoxide and FS enedial (Fig. 10). The relative contribution of each metabolite to covalent binding and toxicity remains unknown.

The major metabolic route of FS in man is glucuronidation. The glucuronide metabolite of FS has been found in human urine and plasma (Beermann et al., 1975, 1977; Benet, 1979; Perez et al., 1979; Smith et al., 1980; Kerremans et al., 1982; Smith and Benet, 1983; Hammarlund-Udenaes and Benet, 1989). We demonstrate the formation of FS-glucuronide in both Wistar rat and CD-1 mouse bile. We confirmed the structure of FS-glucuronide through synthesis of the authentic standard, which coeluted with the glucuronide found in bile (data not shown). Our findings are supported by reports that have identified FS-glucuronide by MS at \( m/z \) [M-H]/H11002 505 and 507 (Mizuma et al., 1998). FS-1-O-acyl glucuronide has been reported to be formed by rat liver microsomes incubated with FS (Rachmel et al., 1985). This report observed an \([M-1]^-\) ion at mass 505, the aglycone fragment at \( m/z \) 329, and the sugar fragment ion of mass 175, as they were in this study. The glucuronide of FS has been shown to be unreactive in terms of covalent binding (Bolze et al., 2002), and compared with other acyl glucuronides, we have observed this using the synthetic FS acyl glucuronide, which remained stable for up to 96 h at physiological pH.

Previous work suggested that an epoxide of FS is responsible for hepatotoxicity observed in the mouse. FS covalently binds to microsomal protein, and the level of binding parallels severity of necrosis. Binding is decreased by cysteine, GSH, and by the presence of P450 enzyme inhibitors, suggesting bioactivation of the FS molecule (Mitchell et al., 1974). Covalent binding was found to be increased in the presence of an epoxide hydrolase inhibitor (Wirth et al., 1976), and no covalent binding was detected with tetrahydrofurosemide, an analog in which the furan ring is reduced and cannot form an epoxide (Wirth et al., 1976).

Demonstration of in vivo metabolism of FS to \( \gamma \)-ketocarboxylic acid and GSH adduct are novel findings with respect to understanding the mechanism of FS toxicity. Both metabolites directly indicate the formation of an epoxide and a \( \gamma \)-ketoenal reactive intermediate. FS can be metabolized to \( \gamma \)-ketocarboxylic acid, which is excreted in bile, which further indicates that FS epoxide is the reactive intermediate. The instability of FS epoxide is evident in the formation of \( \gamma \)-ketocarboxylic acid and the levels of covalent binding observed.
Electrophilicity of FS epoxide results in attacks on nucleophilic macromolecules, hence the covalent binding, which may allow for GSH conjugation. FS has no effect on hepatic GSH levels in vivo. GSH depletion was observed in rat primary hepatocytes. In agreement with the literature, hepatic GSH levels and cytosolic and mitochondrial GSH content were not affected by a 1.21 mmol/kg dose of FS in mice (Mitchell et al., 1974; Wong et al., 2000). Previously, FS was shown to have no effect on GSH content or cell viability in rat hepatocytes (Takagi et al., 1991). Depletion (70%) in cellular GSH has been reported in mouse hepatocytes (Grewal et al., 1991), in agreement with other in vitro mouse hepatocyte studies (Massey et al., 1987). Depletion was observed at 3 h, with lactate dehydrogenase leakage, cytosolic [Ca²⁺] appearance, and protein sulfhydryl depletion being observed at 4 h. This discrepancy could be due to more rapid elimination in vivo. However, in these previous studies, with only 1-h lag between GSH depletion and cytotoxicity, the GSH depletion seen in mice in vitro is a consequence of the cytotoxicity rather than the cause. Importantly, in the previous studies, neither FS metabolism nor bioactivation were assessed.

In rat hepatocytes, which are able to form a GSH conjugate, GSH depletion became significant at 100 μM (6 h) and at 1 mM (1 h), and significant cytotoxicity became evident at 750 μM (6 h) and 1 mM (4 h). This suggests that formation of FS-SG leading to rat hepatocyte GSH depletion was the cause of the in vitro cytotoxicity, along with covalent binding to protein. Murine hepatocytes do not form FS-SG and show no signs of GSH depletion; however, cytotoxicity is observed in murine hepatocytes. We conclude that covalent binding is the cause of cytotoxicity in the murine hepatocyte. In rat hepatocytes, GSH depletion and covalent binding may contribute to cytotoxicity, hence greater cytotoxicity seen in the rat hepatocytes, compared with mouse hepatocytes. In vitro, there is more covalent binding intermediate formed in the rat, compared with mouse. At lower doses (0.5–1 mM) and earlier time points (1–2 h), there are differences in the cytotoxicity between the rat and the mouse. This is attributable to the decreased levels of GSH in the rat hepatocytes, due to GSH conjugation of FS, allowing more conjugation to mouse hepatocyte protein, making them more susceptible to in vitro cytotoxicity. At higher doses and later time points, cytotoxicity is similar between the species, with the rat hepatocytes having greater covalently bound material associated.

To define the ultimate hepatotoxin, we employed the simultaneous assessment of both metabolism and toxicity in vivo with a sensitive and nonsensitive species. FS was shown to be toxic to mice at 5 h, with ALTs significantly increased (Wong et al., 2000), whereas severe toxicity appears at 24 h (Mitchell et al., 1974). However, the same dose of FS in rats had no effect on serum ALT levels. The CYP450 inhibitor, ABT, prevented γ-ketocarboxylic acid formation, indicating formation of γ-ketocarboxylic acid and FS-epoxide is CYP450 mediated. In vivo, there was 6-fold higher covalent binding of FS to mouse liver compared with the rat. ABT reduced, by 14-fold, the level of covalently bound FS in vivo to mouse hepatic protein. ABT protected against FS toxicity, demonstrating that formation of the toxic metabolite is P450-dependent. Table 1 provides an overview of turnover of FS in mouse and rat, alongside levels of covalent binding in vivo.

An important observation is the correlation between irreversible binding to hepatic protein and serum ALT activity, in light of the lack of GSH depletion. In the mouse, FS epoxide can bypass the hepatic GSH pools and bind to protein. In contrast to that seen previously with FS, predepletion of hepatic GSH in mice did not lead to exacerbation of hepatotoxicity (Mitchell et al., 1973). The observation of a GSH conjugate in rats, yet not in mice, may help explain species susceptibility. In addition, the overall turnover to stable metabolites is much greater in the rat (66% turnover) than the mouse (10% turnover). This would lead to a continuously higher hepatic load of FS in the mouse liver compared with the rat liver, allowing covalent binding, initiating cell death.

We incubated FS with NAL and NAC in a mouse liver microsomal system to examine whether FS undergoes N-substituted cysteinyl pyrrole formation, similar to ipomeanol (Baer et al., 2005). The detection of an NAL/NAC-FS adduct in vitro provides further evidence of FS bioactivation and an alternative metabolic pathway to that seen in vivo. In vitro, FS forms a highly unstable δ-ketoenal that can be trapped by equimolar amounts of NAL and NAC, further evidence of epoxide formation.

Screening systems are required to identify the potential of novel compounds to cause toxicity in humans at the earliest stage of drug development. Although FS is not a toxic threat.
to humans, we provide evidence that furan moieties can be bioactivated to epoxide intermediates and that an unsubstituted furan, of the type found in FS, should be regarded as a safety issue. The lack of hepatotoxicity observed in humans with FS will be a function of the extent of epoxidation of the furan ring and the dose of FS required for efficacy (1–2 mg/kg human) relative to the dose required for toxicity [400 mg/kg in mice; compare clozapine with olanzapine (Uetrecht, 2001)]. Certain substructures are considered structural alerts and a potential hazard, and the risk of hepatotoxicity will be a function of dose, fractional clearance of reactive metabolite, efficiency of metabolic biotransformation, and downstream events, such as induction of cell defense. It is imperative that downstream functional biological markers are assessed during preclinical screening, on top of assessment of GSH conjugate formation and/or covalent binding to protein. In the case of FS, in vitro hepatocyte models and the CD-1 mouse are incorrect predictors of the safety profile of FS in humans. The lack of FS-induced hepatotoxicity in man can be explained through a rigorous assessment of both qualitative and quantitative drug metabolism within the target organ, similar to that observed with the hepatotoxicity of tamoxifen in rodents versus humans (Boocock et al., 2000).

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