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METABOLISM AND EXCRETION OF [^{14}C]FURFURAL IN THE RAT AND MOUSE

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Abstract—The fate of furfural (2-furancarboxaldehyde) was investigated in male and female Fischer 344 (F344) rats given single oral doses of 1, 10 and 60 mg kg and male and female CD1 mice given 1, 20 and 200 mg/kg [*carbonyl*-^{14}C]furfural. There was a very high recovery (more than 90% of dose) of radioactivity in all dose groups in 72 hr. The major route of elimination was by the urine, with much smaller amounts present in the faeces and exhaled as $^{14}CO_2$. The residue in the carcass after 72 hr was less than 1% of the administered dose. Furoylglycine and furanacryloylglycine were identified as the major urinary metabolites by high-performance thin-layer chromatography, radio-HPLC, gas chromatography–mass spectrometry and ^{1}H-nuclear magnetic resonance spectroscopy, by comparison with synthetic reference compounds. There were only subtle differences in the metabolic profile as a function of dose size, sex and species. An additional minor polar metabolite was excreted by male rats and mice, and the parent acids of the glycine conjugates were excreted at the higher doses. The results are discussed in terms of the participation of xenobiotics in the chain elongation reactions of fatty acid biosynthesis.

INTRODUCTION

Furfural (2-furancarboxaldehyde) is ubiquitous in nature, and is formed during the thermal decomposition of pentosans in plant materials. It is used extensively in industry, but the most human exposure to furfural is due to its natural occurrence in foods. Some 229,000 kg of furfural are consumed in foods annually, compared with only 2296 kg as an added flavour (Stofberg and Grundschober, 1987). The major foods containing furfural are roasted coffee, alcoholic beverages and white bread (Maarse and Visscher, 1989). Furfural is not inert chemically and exposure to furfural in the workplace has been reported to cause irritation of the mucous membranes of the respiratory and digestive tracts in humans (Bugyi and Lépold, 1951). Experimental studies of furfural toxicity in male Wistar rats have demonstrated hepatic cirrhosis following single or repeated oral administration (Shimizu and Kanisawa, 1986). A long-term carcinogenicity bioassay by the US National Toxicology Program (NTP, 1990) in Fischer rats at doses of 0, 30 and 60 mg/kg in corn oil by gavage (5 days/wk, for 103 wk), provided some evidence of hepatic cholangiocarcinomas and bile duct dysplasia in males but no evidence of carcinogenic activity of furfural in females. There was clear evidence of carcinogenicity in male B6C3F$_1$ mice given 0, 50, 100 and 175 mg furfural/kg by gavage, based on the high incidence of hepatocellular adenomas and carcinomas, and some evidence of carcinogenic activity in females based on the increased incidence of hepatocellular adenomas.

The excessively high, long-term exposure levels used in rodent carcinogenicity bioassays may constitute a flawed basis for judging the potential toxic and/or carcinogenic risk to humans, particularly when human epidemiological data are lacking. However, the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1993) have recommended that the use of furfural be restricted, mainly on the basis of inconsistent reports of genotoxicity in various bacterial and mammalian test systems and the above data on the carcinogenicity of furfural in rodents.

Xenobiotic biotransformation studies in rodent species and humans can generate valuable qualitative and quantitative information about mechanisms contributing to alterations in biochemical and physiological status, particularly informing the extrapolation of high-dose rodent data to the low-dose human situation. The intriguing biotransformation of furfural in the rabbit and dog was first reported by Jaffé and Cohn (1887), who discovered that, in addition to the urinary excretion of furoic acid and furoylglycine, a two-carbon side-chain elongated metabolite, furanacryloylglycine, was a minor metabolite. Hens did not excrete furanacryloylglycine nor its parent acid, but converted furfural

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Abbreviations: BSTFA=*N,O*-bis-trimethylsilyltrifluoroacetamide; *p*-DMAB=*p*-dimethylaminobenzaldehyde; GC-MS=gas chromatography–mass spectrometry; HPTLC = high-performance thin-layer chromatography; NMR = nuclear magnetic resonance; TMCS = trimethylchlorosilane; TMS = trimethylsilyl.

to difuroylornithine (Jaffé and Cohn, 1888): in galliform birds, the glycine conjugation mechanism is replaced by one involving ornithine (Caldwell *et al.*, 1980).

The subsequent history of this early finding of a novel biosynthesis is noteworthy. Furanacryloylglycine was not detected as a metabolite in rats (Irwin *et al.*, 1985; Jodynis-Liebert and Laboda, 1982; Nomeir *et al.*, 1991; Paul *et al.*, 1949) but was found in the urine of humans following exposure to furfural vapour (Flek and Sêdivec, 1978). Irwin *et al.* (1985) and Nomeir *et al.* (1991) have both reported the formation of furanacrylic acid by rats given furfural, in addition to furoic acid and furoylglycine. Furoylglycine is an endogenous component in the urine of healthy adults, but not of a 1-month-old breast-fed child nor various experimental animals (Pettersen and Jellum, 1972). Furoylglycine has also been detected in the urine of adult humans following the ingestion of furoic acid as its sodium salt (Schempp, 1921).

The purpose of this investigation was to establish, using current methodology, the metabolic fate of furfural in Fischer 344 rats and CD1 mice, and the influence of sex and dose size on this.

MATERIALS AND METHODS

Materials

[*carbonyl*-^{14}C]Furfural, specific activity 135.75 μCi/mg (radiochemical purity 98.1% by radio-gas chromatography) was a custom synthesis by Amersham International plc (Amersham, Bucks., UK) and was diluted to constant specific activity 29.5 μCi/mg with unlabelled furfural (Aldrich Chemical Co., Gillingham, Dorset, UK) and stored in dichloromethane in a glass vial at a temperature of −20 C. Radiochemical purity was more than 99% by radio-HPLC (system 1).

Furoic acid, furoyl chloride, furanacrylic acid, 2-acetylfuran, furfuryl alcohol and *N*-methyl-*N*-nitrosotoluene-*p*-sulfonamide for the synthesis of diazomethane were purchased from Aldrich. Trioctanoin and enzymes used for the treatment of urine samples were obtained from Sigma Chemical (Poole, Dorset, UK). *N,O*-bis-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS) was purchased from Pierce and Warriner (Chester, UK). General laboratory reagents were obtained from usual commercial sources and were either analytical grade or HPLC grade.

Furoylglycine. Furoyl chloride (5 g) was added dropwise to 10% excess glycine (3.16 g) in 50 ml 1 M sodium hydroxide solution with stirring. Stirring was maintained for 4 hr until the reaction was complete. The reaction mixture was acidified to pH 1 with concentrated HCl and the furoylglycine so precipitated was collected by filtration. Contaminating furoic acid was removed by the addition of cold diethyl ether. The crude product was recrystallized twice from ethanol, yield 0.98 g, 15% of theory, m.p. 171 C (literature value 165 C; Jaffé and Cohn, 1887; Sasaki, 1910). Mass spectrometry (MS) [by gas chromatography–mass spectrometry (GC–MS) as methyl ester]: m/z 183 (M^+), 124 (M-$COOCH_3^+$), 95 (M-$NHCH_2COOCH_3^+$), 67 ($C_4H_3O^+$); ^{1}H-nuclear magnetic resonance (NMR): δ (ppm) 4.16 (s, -CON-HCH_2COOH), 6.65 (q, furan C^{4}H), 7.22 (d, furan C^{3}H), 7.71 (d, furan C^{5}H).

Furanacryloylglycine. Glycine ethyl ester hydrochloride (14.3 g) was dissolved in water (50 ml) and shaken with 4 M sodium hydroxide solution (25 ml) and diethyl ether (500 ml). The layers were separated and the aqueous phase discarded. The ether phase was dried (anhydrous Na_2SO_4) and evaporated *in vacuo*. The glycine ethyl ester (3.9 g) was dissolved in 1,4-dioxan (75 ml) and furanacrylic acid (5.1 g) added. *N,N'*-dicyclohexylcarbodiimide (7.6 g) was then added and the reaction mixture was continuously stirred for 24 hr. Glacial acetic acid (5 ml) was added and the precipitated *N,N'*-dicyclohexylurea removed by filtration. The 1,4-dioxan was evaporated under reduced pressure and the residue was dissolved in 0.2 M NaOH (100 ml) and heated in a water-bath at 90 C for 1 hr. The solution was acidified with concentrated HCl (10 ml) and the furanacryloylglycine collected by filtration. The product was washed twice with cold diethyl ether (20 ml) and recrystallized twice from hot ethanol [yield 0.6 g (8.6% of theory)]. When heated, the crystalline product darkened in colour at 207 C, and then melted into a dark brown liquid at 226 C (literature value 208–215 C, Jaffé and Cohn, 1887; 215–219 C, Sasaki, 1910). MS (by GC–MS as methyl ester) m/z 209 (M^+), 178 (M-OCH_3^+), 121 (M-$NHCH_2COOCH_3^+$); ^{1}H-NMR: δ (ppm) 4.01 (s, -CON-HCH_2COOH), 6.49 (coalesced q, furan C^{4}H and d, furan-CH=CH), 6.67 (d, furan C^{3}H), 7.32–7.35 (d, furan-CH=CH, J=15 Hz), 7.58 (d, furan C^{5}H).

Animal studies

Fischer 344 rats (Harlan OLAC Ltd, Blackthorn, Bicester, Oxon, UK) and CD1 mice (Charles River UK Ltd, Margate, Kent, UK) were allowed to acclimatize for 5 days following arrival in an air-conditioned room at 21 ± 2 C with a 12-hr light/dark cycle. 1 day before dosing the animals were individually housed in glass metabolism cages [Metabowls (rats) and MiniMetabowls (mice); Jencons Ltd (Leighton Buzzard, Beds., UK)] to permit the separate collection of urine, faeces and expired air. The animals were maintained on a pelleted diet (Labsure CRM, Biosure Ltd, Manea, Cambridge, UK) and tap water *ad lib.* for the duration of the experiment.

Male (body weight 180–220 g) and female (150–180 g) Fischer 344 rats were given single doses by oral gavage of 1, 10 and 60 mg [^{14}C]furfural/kg, 10 μCi per rat (five animals per group). Male (25–30 g) and female (20–25 g) CD1 mice were given

single doses by oral gavage of 1, 20 and 200 mg [^{14}C]furfural/kg in trioctanoin by oral gavage, 6 μCi per mouse (five animals per group).

Excreta were collected at 0–24, 24–48 and 48–72 hr after dosing. Urine was collected in glass vessels cooled by ice. In certain mouse studies, expired air was drawn through two traps of Carbosorb E (Canberra Packard, Pangbourne, Berks, UK) to trap $^{14}CO_2$.

Urine was weighed, immediately counted for radioactivity and stored in glass vials at −20°C until further analysis. The cages were washed with distilled water daily and the ^{14}C content of the washings added to the urinary recovery. Faeces were weighed, homogenized in three volumes of water and stored at −20°C until analysed. The animals were killed by cervical dislocation 72 hr after dosing and the carcasses stored at −20°C.

Radiochemical techniques

Liquid scintillation spectrometry was performed using either Packard TriCarb 4640 or Minaxi TriCarb 4000 Series instruments (Canberra Packard) with external standardization for quench correlation. Total ^{14}C in urine samples, cage washings and dose solutions was assayed by directly counting of triplicate weighed aliquots (25–1000 μl) using 3 ml Ecoscint (National Diagnostics, Watford, UK).

The ^{14}C content of rat and mouse faecal samples was determined by a modification of the procedure outlined by Caldwell *et al.* (1972). Faeces were homogenized in water for 1 min using a Lab-Blender 80 stomacher (Seward Medical, London, UK). A weighed aliquot of the faecal homogenate (approx. 1–1.5 g) was mixed well with 2 ml 100 vol H_2O_2, 0.2 ml isoamyl alcohol to control foaming and 1 ml 5 M NaOH solution in loosely stoppered, foil-wrapped test-tubes and left at room temperature overnight. The mixture was neutralized with 0.2 ml glacial acetic acid, and the volume was made up to 10 ml with ethanol. The mixture was heated to 65°C in a water-bath for 45 min to remove residual H_2O_2, and cooled. The volume was made up to 10 ml with ethanol and the mixture centrifuged. Triplicate 0.5 ml aliquots of the supernatant were placed in polyethylene vials and 10 ml Ecoscint was added. The capped vials were left to stand for 4 hr before counting, to minimize chemiluminescence. A quench correlation curve was established using the faeces collected from an untreated animal.

The ^{14}C remaining in the carcasses was quantificated following digestion. The frozen carcasses were cut into 2 cm^3-cubes with secateurs and placed in 40% (w/v) NaOH in 80% (v/v) aqueous ethanol (1 ml/g of carcass) in glass jars. The mixtures were left to digest completely for 10 days in a fume hood at room temperature. Triplicate 1-ml aliquots of the digest were mixed with 0.1 ml iso-amyl alcohol and 0.2 ml 100 vol H_2O_2. After 2 hr, 0.2 ml concentrated hydrochloric acid and 20 ml Ecoscint were added. The capped vials were left to stand for 4 hr prior to counting so as to reduce chemiluminescence. A quench correlation curve was established using the carcass of an untreated animal.

For the determination of exhaled $^{14}CO_2$, 15 ml Permafluor V, a pseudocumene based scintillator (Canberra Packard) was added to polyethylene vials containing 1-ml aliquots of Carbosorb E trapping solution.

High-performance thin-layer chromatography

Aliquots of 0–24 hr pooled urine from male and female rat (60 mg/kg), and male and female mouse (200 mg/kg) high dose groups, and the appropriate pre-dose urine pools from each group were centrifuged at 11,000 rpm for 5 min. The urine supernatants were spotted onto pre-coated silica gel 60 F_{254} high-performance thin-layer chromatography (HPTLC) plates (0.2 mm thick layer, 20 × 20 cm aluminium support, cat. no. 5548, E. Merck, Darmstadt, Germany) which were used developed with ethyl acetate–glacial acetic acid–water (35:1:1, by vol) by the ascending technique to about 16 cm from the origin. The various compounds were located as dark fluorescence-quenching spots when the dried chromatograms were viewed under UV light (254 nm). To detect glycine conjugates, the chromatograms were sprayed with a 10% solution (w/v) of *p*-dimethylaminobenzaldehyde (*p*DMAB) in acetic anhydride. Following heating of the chromatograms at 120°C for 5 min, glycine conjugates were viewed as red/orange spots (El Masry *et al.*, 1956). R_f values of compounds of interest in this system were as follows: furoic acid, 0.49; furoylglycine, 0.20; furanacrylic acid, 0.69 and furanacryloylglycine, 0.23.

High-performance liquid chromatography with online radioactivity detection (Radio-HPLC)

Chromatographic separations were performed using two Shimadzu LC-6A pumps, an SCL-6A solvent gradient programmer, an SIL-6A autoinjector and an SPD-6A UV detector set at 254 nm (supplied by Dyson Instruments, Houghton-le-Spring, Tyne and Wear, UK). The system was coupled to a Ramona 90 radioactivity flow monitor with a solid yttrium silicate cell. Data were processed with the Ramona chromatographic system package (Raytek Scientific Ltd, Sheffield, UK) installed on an Elonex PC-420X computer.

System 1. This used a LiChrosorb RP8 pre-packed guard column and analytical column 5 μm particle size, 25 cm × 4.0 mm i.d. (Hichrom Ltd, Reading, Berks, UK). Solvent A: degassed water containing 1% (v/v) glacial acetic acid; solvent B: degassed acetonitrile containing 1% (v/v) glacial acetic acid. A linear solvent gradient system was used, as shown in Table 1. Flow rate was 1 ml/min throughout.

System 2. This system was optimized to prevent both ionization of acidic metabolites and co-elution with endogenous hippuric acid in urine. An Excel

Table 1. Linear solvent gradient system used in radio-HPLC system 1

Solvent*	Percentage of solvent used at x min			
	$x = 0$	30	35	40
A	95	85	65	95
B	5	15	35	5

*Solvent A = degassed water containing 1% (v v) glacial acetic acid; solvent B = degassed acetonitrile containing 1% (v/v) glacial acetic acid.

Range Spherisorb S50DS2 guard column and analytical column, 5 μm particle size, 25 cm × 4.6 mm i.d. (Hichrom Ltd) were used. Solvent A: degassed water containing 1% (v/v) glacial acetic acid; solvent B: degassed methanol containing 1% (v/v) glacial acetic acid. A linear solvent gradient system was used, as shown in Table 2. The flow rate was 1 ml/min throughout.

Gas chromatography–mass spectrometry

GC–MS used an HP5890 Series II gas chromatograph with an HP5971 Series mass selective detector and HP G1034C software for the MS ChemStation running on an HP Vectra 486N computer (Hewlett Packard, Stockport, Cheshire, UK). The column was an HP fused silica capillary (30 m × 0.25 mm i.d.) coated with cross-linked 5% phenyl methyl silicone (film thickness 0.25 μm, phase ratio 250) with helium as the carrier gas (flow rate 1 ml/min). The injection port temperature was 250 C and the transfer line temperature was 280 C. The column temperature programme was 2 min at 80 C, increased at 5 C/min to a final temperature of 250 C and held at 250 C for 10 min. A 1-μl sample was injected manually by split injection or splitless injection with the split closed for 1 min. The MS used a 70 eV electron impact ion source and the scanned mass range was 45–550, scan rate 1.5/sec. The solvent delay was 6 min for analysis of trimethylsilyl (TMS) derivatives and 1.8 min for analysis of diazomethane-treated derivatives. The voltages of the repeller, draw out, ion focus, entrance lens and X-ray and the parameters for the quadrupole mass filter were set with the autotune programme, which optimizes these parameters using perfluorotributylamine as a calibrant. The probability-based matching library searching in the MS ChemStation software facilitated the verification of spectra from derivatized ^{14}C metabolites against spectra of TMS derivatives or methyl esters of reference compounds.

^{1}H-Nuclear magnetic resonance spectroscopy

^{1}H-NMR spectra were recorded at 500 MHz with the Bruker WT500 instrument of the Department of Chemistry, Imperial College of Science, Technology and Medicine. Chemical shifts are expressed relative to the solvent signal (D_2O or d_4-methanol).

Incubation of urine with 1 M NaOH

The proportion of mild alkali-resistant and labile ^{14}C metabolites was ascertained, based on the premise that acyl glucuronides are labile and amino acid conjugates are stable to such treatment (Caldwell and Hutt, 1986). Pooled 0–24 hr urine from the highest dose groups of male and female rats and mice were treated with 1 M sodium hydroxide solution (pH 10) for 4 hr at room temperature. An untreated pooled pre-dose urine sample served as a control. A mixture of reference compounds (furoic acid, furanacrylic acid, benzoic acid, furoylglycine, furanacryloylglycine and hippuric acid) dissolved in 20% aqueous methanol was treated in the same manner to serve as an additional control. After 4 hr, the samples were neutralized with 1 M HCl, centrifuged at 11,000 rpm for 5 min, and the supernatants analysed by radio-HPLC (system 2).

Incubation of urine with β-glucuronidase or sulfatase

Pooled 0–24-hr urine samples from the highest dose groups of male and female rats and mice were treated in the following manner:

Table 2. Linear solvent gradient system used in radio-HPLC system 2

Solvent*	Percentage of solvent used at x min									
	$x = 0$	3	16	18	26	30	36	46	50	65
A	96	94	94	89	80	50	50	0	0	96
B	4	6	6	11	20	50	50	100	100	4

*Solvent A = degassed water containing 1% (v v) glacial acetic acid; solvent B = degassed methanol containing 1% (v v) glacial acetic acid.

Table 3. Elimination of radioactivity by male and female Fischer 344 rats given single oral doses of [^{14}C]furfural

Sex	Dose level (mg kg)	Percentage recovery of dose administered							
		0–24 hr		24–48 hr		48–72 hr			
		Urine	Faeces	Urine	Faeces	Urine	Faeces	Carcass	Total
Male	1	95.9 ± 2.4	3.4 ± 1.9	1.2 ± 0.9	0.3 ± 0.1	0.8 ± 0.3	0.1 ± 0	0.5 ± 0.2	102.3 ± 1.8
	10	87.0 ± 3.2	3.6 ± 3.2	0.6 ± 0.3	0.5 ± 0.4	2.5 ± 2.4	0.1 ± 0.2	0.5 ± 0.1	94.9 ± 1.9
	60	87.9 ± 2.0	2.0 ± 2.1	0.6 ± 0.2	0.9 ± 0.4	0.5 ± 0.2	0.2 ± 0.1	0.5 ± 0.2	92.6 ± 1.2
Female	1	76.3 ± 3.7	6.1 ± 1.8	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.1 ± 0	1.0 ± 0.3	83.8 ± 7.1
	10	83.8 ± 8.1	3.4 ± 3.1	0.7 ± 0.4	0.6 ± 0.4	1.2 ± 0.7	0.1 ± 0	0.6 ± 0.2	90.4 ± 4.7
	60	76.0 ± 15.3	3.4 ± 0.9	0.7 ± 0.3	0.6 ± 0.3	0.7 ± 0.5	0.2 ± 0.3	0.6 ± 0.1	82.1 ± 19.9

Values are means ± SD, n = 5.

1. Urine mixed with an equivalent volume of 0.2 M acetate buffer, pH 5;
2. Urine:glucurase solution:0.2 M acetate buffer, pH 5 (2:1:1, by vol);
3. Urine:sulfatase solution:0.2 M acetate buffer, pH 5 (2:1:1, by vol) containing 1.5 mg D-saccharic acid 1,4-lactone/ml to inhibit the β-glycuronidase activity present in the sulfatase preparation.

The final volume of the above mixtures was 600 μl. They were incubated at 37 C in a shaking water-bath for 24 hr, with positive and negative controls using *p*-nitrophenyl glucuronide and sulfate essentially as described by Caldwell and Hutt (1986).

Samples were centrifuged (11,000 rpm, 5 min) and the supernatants profiled by HPLC in system 2.

Isolation and identification of ^{14}C urinary metabolites

Pooled 0–24-hr urine samples from the highest dose groups of male and female rats and mice were centrifuged (11,000 rpm for 5 min) and freeze-dried. The residues were exhaustively extracted with 97% acetonitrile until 95–98% of the original ^{14}C content was recovered. The extracts were dried under a stream of dry N_2 and reconstituted in 20% aqueous methanol to give a three-fold concentration of urinary radioactivity. Aliquots from each pool were examined by radio-HPLC (system 2) and ^{14}C metabolites isolated by collecting the peak of interest on the radio-HPLC chromatogram in glass vials, from repeated injections of the sample. Organic solvents in the HPLC eluate collections were evaporated under a stream of dry N_2 and freeze-dried.

The dried residues were either taken up in deuterated solvents for NMR, treated with BSTFA + 1% TMCS (100 μl) for 3 min at room temperature, with vortexing, to form TMS derivatives or taken up in 20 μl acetonitrile and treated with 1 ml ethereal diazomethane for 10 min. Excess reagents were evaporated under N_2 and the TMS derivatives and methyl esters so formed were examined by GC–MS.

Samples of pooled pre-dose urine from the rat and mouse high dose groups were carried through the same procedure.

RESULTS

Recovery of radioactivity

The excretion of radioactivity by rats and mice after single oral gavage doses of [*carbonyl*-^{14}C]furfural is presented in Tables 3 and 4, respectively. An essentially quantitative recovery was achieved in all groups in 72 hr. The main route of elimination was the urine (more than 60% within the first 24 hr). Faecal elimination (3–7% in 72 hr) and expired $^{14}CO_2$ (in a limited number of CD1 mouse studies; 5% in 72 hr) constituted minor routes of excretion. The carcasses of both species contained less than 1% of the ^{14}C dose after 72 hr.

Table 4. Elimination of radioactivity in male and female CD1 mice given single oral doses of ^{14}C-furfural

Sex	Dose level (mg/kg)	Percentage recovery of dose administered									
		0–24 hr			24–48 hr			48–72 hr			
		Urine	Faeces	$^{14}CO_2$	Urine	Faeces	$^{14}CO_2$	Urine	Faeces	$^{14}CO_2$	Carcass	Total
Male	1*	61.3 ± 10.4	4.2 ± 3.4	—†	9.4 ± 2.8	1.5 ± 1.6	—	5.1 ± 3.0	0.1 ± 0.2	—	0.5 ± 0.3	80.3 ± 3.5
	20	91.4 ± 3.7	2.5 ± 0.9	—	0.9 ± 0.8	0.2 ± 0.2	—	0.7 ± 0.4	0	—	0.4 ± 0.1	96.1 ± 3.4
	200	90.0 ± 10.0	2.3 ± 0.3	5.2 ± 0.2‡	7.8 ± 1.5	0.9 ± 1.1	0.2 ± 0.1	6.8 ± 5.2	0.2 ± 0.2	—	0.7 ± 0.3	108.3 ± 8.9
Female	1	73.8 ± 9.2	1.3 ± 0.7	4.2 ± 0.2	7.3 ± 2.7	0.4 ± 0.2	0.2 ± 0	3.4 ± 1.4	0.2 ± 0.1	—	0.3 ± 0.1	88.6 ± 7.5
	20	87.5 ± 8.1	2.9 ± 1.9	—	1.9 ± 0.8	0.2 ± 0.3	—	1.9 ± 2.6	0	—	0.5 ± 0.4	94.2 ± 7.4
	200	72.4 ± 12.4	1.0 ± 1.1	—	9.4 ± 3.0	0.1 ± 0	—	3.9 ± 5.3	0	—	0.6 ± 0.1	87.4 ± 9.9

*Mean recovery from four mice.
†$^{14}CO_2$ not collected.
‡Mean $^{14}CO_2$ recovery from two mice.
Values are means ± SD, n = 4.5.

Table 5. Gas chromatographic and diagnostic mass spectral data of metabolites of furfural and of reference compounds

Compound	GC R_t (min)	m/z (percentage relative abundance)
Furoylglycine mono-TMS	21.2	241 (3), 226 (2), 196 (12), 169 (24), 143 (14), 95 (100), 73 (63)
Furoylglycine bis-TMS	21.5	313 (3), 296 (34), 270 (16), 196 (100), 147 (29), 95 (25), 73 (57)
Metabolite B mono-TMS	21.3	241 (3), 226 (28), 196 (8), 169 (17), 143 (12), 95 (100), 73 (75)
Metabolite B bis-TMS	21.6	313 (2), 296 (25), 196 (94), 147 (31), 95 (36), 73 (100)
Furoylglycine methyl ester	17.6	183 (15), 124 (40), 95 (100), 67 (3)
Metabolite B methyl ester	17.7	183 (14), 124 (40), 95 (100), 67 (3)
Furoic acid TMS	7.8	184 (12), 169 (62), 126 (12), 125 (100), 95 (27)
Metabolite C TMS	7.8	184 (11), 169 (59), 126 (12), 125 (100), 95 (33)
Furoic acid methyl ester	4.3	126 (33), 95 (100), 67 (4)
Metabolite C methyl ester	4.3	126 (35), 95 (100), 67 (4)
Furanacryloylglycine mono-TMS	28.3	267 (25), 252 (6), 222 (5), 178 (4), 149 (7), 121 (100), 73 (35), 65 (14)
Furanacryloylglycine bis-TMS	27.5	339 (34), 324 (10), 285 (6), 222 (39), 206 (12), 147 (10), 121 (100), 73 (41)
Metabolite D mono-TMS	28.4	267 (27), 252 (6), 222 (7), 178 (4), 149 (6), 121 (100), 73 (40), 65 (21)
Metabolite D bis-TMS	27.5	339 (21), 324 (6), 285 (4), 222 (29), 206 (9), 147 (8), 121 (100), 73 (52)
Furanacryloylglycine methyl ester	25.3	209 (27), 178 (2), 121 (100), 65 (24)
Metabolite D methyl ester	25.3	209 (26), 178 (2), 121 (100), 65 (23)
Furanacrylic acid TMS	14.3	210 (39), 195 (67), 151 (86), 121 (100), 65 (22)
Furanacrylic acid methyl ester	9.7	152 (53), 121 (100), 65 (42)

GC = gas chromatography TMS = trimethylsilane

Table 6. Quantitative radio-HPLC (system 2) metabolite profile of 0–24 hr urine from Fischer 344 rats

	Percentage of ^{14}C metabolites at various dose levels (mg/kg)					
	Males			Females		
Metabolite	1	10	60	1	10	60
Furfural	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Unknown A	2.4 ± 1.3	2.9 ± 1.1	2.8 ± 0.8	n.d.	n.d.	n.d.
Furoic acid	n.d.	n.d.	2.2 ± 1.6	n.d.	n.d.	1.9 ± 0.8
Furoylglycine	81.5 ± 3.3	81.8 ± 3.5	79.3 ± 3.8	76.2 ± 5.9	83.8 ± 4.5	79.9 ± 10.2
Furanacrylic acid	n.d.	n.d.	n.d.	n.d.	n.d.	2.4 ± 0.2
Furanacryloylglycine	17.0 ± 2.3	15.3 ± 2.6	16.2 ± 4.8	23.8 ± 5.9	16.2 ± 4.5	17.4 ± 8.1

n.d.=not detected

Values are means ± SD, n=5, of ^{14}C present in 0–24 hr urine.

Table 7. Quantitative radio-HPLC (system 2) metabolite profile of 0–24 hr urine from CD1 mice

	Percentage of ^{14}C metabolites at various dose levels (mg/kg)					
	Males			Females		
Metabolite	1	20	200	1	20	200
Furfural	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Unknown A	n.d.	n.d.	1.4 ± 0.3	n.d.	n.d.	n.d.
Furoic acid	n.d.	n.d.	2.1 ± 1.6	n.d.	4.4 ± 4.5	10.4 ± 2.8
Furoylglycine	85.7 ± 4.5	89.3 ± 2.0	84.4 ± 4.2	65.0 ± 8.7	79.2 ± 7.0	76.0 ± 3.6
Furanacrylic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Furanacryloylglycine	14.3 ± 4.5	10.7 ± 2.0	12.0 ± 3.3	35.0 ± 8.7	16.4 ± 7.1	13.6 ± 3.1

n.d. = not detected

Values are means ± SD, n = 5, of ^{14}C present in 0–24 hr urine.

Analysis of urinary metabolites

Furoylglycine (R_F 0.20) and furanacryloylglycine (R_F 0.23) were detected as positive (red/orange) spots with the *p*DMAB spray on HPTLC plates in urine from rats and mice at the highest dose levels in both sexes.

Radio-HPLC analysis of urine showed that [^{14}C]furfural is completely metabolized by rats and mice at all dose levels and no unchanged compound is excreted in the urine. More than 98.7% of the ^{14}C injected onto the HPLC column was recovered in the five distinct ^{14}C peaks seen and there was no appreciable change in the radio-HPLC profiles (system 2) after treatment with 1 M NaOH for 4 hr at room temperature. Similarly, there was no evidence for the presence of β-glucuronidase or sulfatase-labile metabolites in urines at the highest dose levels in both sexes and species, as the radio-HPLC metabolite profiles (system 2) were unaltered.

Identification of metabolites

The five ^{14}C-metabolites were isolated from urine and characterized by physicochemical techniques.

Metabolite A (R_t 4.0 min; 2% in male rats; 1% in high dose male mice) is not a glucuronide or a sulfate. The nature of this very polar metabolite remains unknown as attempts to isolate sufficient materials for spectral identification were unsuccessful.

Metabolite B (approx. 80% of dose in rats and mice) was identified as furoylglycine by a red/orange spot on HPTLC with *p*DMAB spray (R_F 0.20) and comparison of HPLC retention (R_t 13.0 min) with the

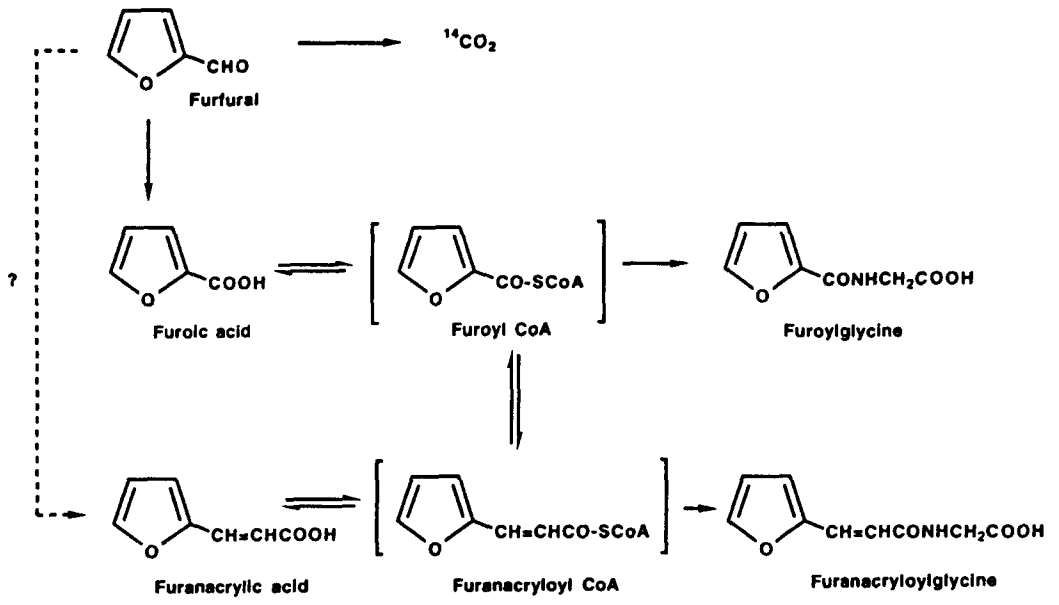

Fig. 1. Metabolic map of furfural describing its fate in the rat and mouse.

authentic standard. Its ^{1}H-NMR spectrum showed δ (ppm) 4.16 (s, -CONHCH$_2$COOH), 6.65 (q, furan C^{4}H), 7.22 (d, furan, C^{3}H), 7.71 (d, furan C^{5}H), all signals identical with those of the synthetic standard. The derivatization of this metabolite with BSTFA + 1% TMCS or diazomethane generated products with identical GC retention and mass spectra to the corresponding derivatives of furoylglycine (Table 5). The diagnostic mass spectral ions of the mono- and bis-TMS derivatives of furoylglycine seen here were in close agreement with those reported in other work (Tjoa and Fennessey, 1979), which described possible rearrangements (Fennessey and Tjoa, 1980) of a series of acylglycine conjugates.

Metabolite C (approx. 2% of dose in high dose rats and male mice; up 10% in female mice) was identified as furoic acid by comparison of its HPLC retention (R_f 14.1 min) with a reference standard. The derivatization of this metabolite by silylation or with diazomethane generated products with mass spectra similar to that of the derivatized products of furoic acid (Table 3).

Metabolite D (10–35% of dose) was assigned to furanacryloylglycine by its red/orange colour with *p*DMAB spray on HPTLC (R_F 0.23) and HPLC retention (R_t 34.5 min), both identical with the synthetic standard. The mass spectra obtained by GC–MS of its mono- and bis-TMS and methyl ester derivatives were essentially identical with those for the authentic furanacryloylglycine (Table 5). Its ^{1}H-NMR spectrum showed δ (ppm) 4.01 (s, -CONHCH$_2$COOH), 6.49 (coalesced q, furan C^{4}H and d, furan-CH=CH), 6.67 (d, furan C^{3}H), 7.32–7.35 (d, furan-CH=CH, J=15 Hz), 7.58 (d, furan C^{5}H), all signals identical with those of the synthetic standards.

Metabolite E (2% of dose in high dose female rats only) has tentatively been assigned as furanacrylic acid based only by comparison of its HPLC retention (R_t 38.0 min) with the authentic standard. Incomplete characterization was due to unsuccessful attempts to isolate sufficient material.

HPTLC and radio HPLC showed furoylglycine and furanacryloylglycine as major metabolites appearing at all dose levels in both sexes of rat and mouse. At higher dose levels, furoic acid appeared in the metabolite profile, particularly in female mice. Furanacrylic acid was excreted by female rats at the 60 mg/kg dose level. In addition, a very minor, polar metabolite (metabolite A), was detected in the urine of male rats at all dose levels and male mice at 200 mg/kg, but could not be identified.

The urinary metabolites of furfural in rats and mice and the influence of dose size and sex are summarized in Tables 6 and 7, respectively.

DISCUSSION

The principal route of excretion of radioactivity after oral gavage administration of [*carbonyl*-^{14}C]furfural to Fischer 344 rats and CD1 mice was the urine. In addition, much smaller amounts were recovered in faeces and carcasses, showing that little bioaccumulation occurred. In CD1 mice, the exhalation of $^{14}CO_2$ indicates that the aldehyde moiety of furfural is oxidized and subsequently decarboxylated. This has been shown in *Pseudomonas putida* Ful which degrades furfural aerobically to 2-furoyl CoA by way of furoic acid, followed by hydroxylation of 2-furoyl CoA to form 5-hydroxy-2-furoyl-CoA. Ring opening leads to 2-oxoglutarate, which enters the tricarboxylic acid cycle giving CO_2 and various other

intermediary metabolites (Koenig and Andreesen, 1990; Trudgill, 1976). The occurrence of a comparable sequence of reactions in rodents would account for the present finding of $^{14}CO_2$: there is evidence for the first two steps in both rats and mice.

The major urinary metabolites have been characterized by HPTLC, radio-HPLC, GC–MS and ^{1}H-NMR as furoylglycine and furanacryloylglycine, accompanied by furoic acid and furanacrylic acid. There was an additional very polar metabolite in males and the excretion of the parent acids of the glycine conjugates increased with increasing dose. It is generally accepted that many aromatic aldehydes and alcohols (e.g. benzaldehyde, vanillin, ethylvanillin) undergo oxidation to their corresponding acids *in vivo* (Williams, 1959). In accordance with this, furfural is oxidized to furoic acid in Fischer 344 rats and CD1 mice, and excreted in the urine principally as furoylglycine. The increased excretion of the free acids at higher dose levels indicates that glycine conjugation was capacity limited, probably by the supply of endogenous glycine for conjugation (Arnstein and Neuberger, 1951; Gregus *et al.*, 1993). Friedmann (1911) reported the urinary excretion of furoic acid by dogs given furanacrylic acid, suggesting that there is a dynamic equilibrium existing between the two acids, most likely at the level of their CoA thioesters, *in vivo*. A comparable equilibrium exists between benzoic and cinnamic acids (Nutley, 1991; Nutley *et al.*, 1994). This equilibrium appears to lie in the direction of furoic acid, accounting for greater excretion of furoylglycine as compared with furanacryloylglycine. However, our knowledge of enzymic and other factors determining such equilibria is scant.

This work provides a confirmation of early findings (Jaffé and Cohn, 1887) that furfural is metabolized to a relatively lipophilic two-carbon elongated product, furanacrylic acid, which is excreted in urine as its glycine conjugate. Jaffé and Cohn (1887) originally proposed the hypothesis that the two-carbon side-chain elongation occurred by the Perkin reaction, that is an aldol condensation of furfural with acetate. It now seems more likely that furanacrylic acid is formed by way of furoic acid which reacts, as furoyl-CoA with acetyl-CoA and/or malonyl-CoA, in the same sequence as the chain elongation of fatty acids, a mechanism proposed to explain urinary excretion of 3-keto-3-phenylpropionic acid and acetophenone as metabolites of benzoic acid in the rat and the horse (see Caldwell and Marsh, 1983; Caldwell and Parkash, 1993; Marsh *et al.*, 1982; Nutley, 1991). The excretion of furoylglycine provides excellent indirect evidence for the formation of furoyl-CoA, since such acyl CoAs are obligatory intermediates in the amino acid conjugations (Hutt and Caldwell, 1991). It has been suggested that the amino acid conjugations protect against the cellular consequences of xenobiotic acyl-CoA formation for lipid biochemistry by acting as 'acyl scavengers' (Caldwell *et al.*, 1980).

The metabolism of furfural in rats and mice is summarized in the metabolic map in Fig. 1. The toxicological significance of possible biochemical perturbations resulting from the chain-elongated metabolites, as suggested by us elsewhere (Caldwell and Parkash, 1993), remains to be explored.

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