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Identification of a *cis*-2-butene-1,4-dial-derived glutathione conjugate in the urine of furan-treated rats

Lisa A. Peterson*, **Meredith E. Cummings**, **Jacqueline Y. Chan**, **Choua C. Vu**, and **Brock A. Matter**
Division of Environmental Health Sciences and the Cancer Center, University of Minnesota, Minneapolis, MN 55455

Abstract

The hepatocarcinogen and toxicant, furan, requires metabolic activation to elicit its toxic effects. The available experimental evidence indicate that the overall metabolism of furan is initiated via cytochrome P450 catalyzed oxidation to *cis*-2-butene-1,4-dial. This α,β-unsaturated dialdehyde reacts in vitro with protein and DNA nucleophiles. To determine if this compound is an in vivo intermediate in the metabolism of furan, rats were treated with either [$^{12}C_4$]furan or [$^{13}C_4$]furan and urine was collected for 24 h. Capillary LC/MS/MS analysis of the urine indicated that one of the metabolites was a mono-glutathione conjugate of *cis*-2-butene-1,4-dial. These results indicate that glutathione conjugation of the reactive metabolite of furan occurs in vivo. This metabolite may serve as a useful marker for furan exposure and metabolism in risk assessment studies.

Keywords

furan; metabolism; stable isotope methods; capillary LC-MS; urinary metabolites

Introduction

Furan is an important industrial chemical that is also present in the environment (1). It is both hepatoxic and carcinogenic after oral administration in mice and rats (2,3). Based on these results and the large potential for human exposure, furan has been listed as a possible human carcinogen (Group 2B) by the National Toxicology Program and the International Agency for Research on Cancer (1,4). However, risk assessment has been hampered by the lack of effective biomarkers for furan uptake and metabolism.

The mechanism of furan induced toxicity and carcinogenicity is unknown. The available experimental data indicate that the toxic effects of furan are initiated by a cytochrome P-450 catalyzed oxidation of furan. Cytochrome P-450 inducers increase furan metabolism and its associated toxic effects such as furan derived protein binding, glutathione (GSH) depletion and cytotoxicity (5-9). Similarly, these toxic effects are reduced in the presence of cytochrome P-450 inhibitors (5–9). The initial product of microsomal furan oxidation is *cis*-2-butene-1,4-dial (Scheme 1) (10–12). This reactive α,β-unsaturated dialdehyde is likely to be a candidate for the ultimate toxic and carcinogenic properties of furan since it readily reacts with protein and DNA nucleophiles (11,13,14) and is a bacterial mutagen (15). The formation and fate of this molecule in vivo has not been investigated.

*To whom requests for reprints should be addressed at The Cancer Center, University of Minnesota, Mayo Mail Code 806, 420 Delaware St. S.E., Minneapolis, MN 55455. Phone: 612-626-0164; fax: 612-626-5135; email: peter431@umn.edu.

Furan is extensively metabolized in rats since only 14% of an 8 mg/kg oral dose of [^{14}C]furan is eliminated unchanged (5). The major metabolite is carbon dioxide, accounting for 26% of the dose. A majority of the remainder is excreted in urine (22%) or feces(20%). Since the uptake of furan is a single saturable process that can be blocked by the cytochrome P450 inhibitor pyrazole (9), a cytochrome P450 catalyzed reaction is thought to initiate the in vivo metabolism of furan. Therefore, the observed microsomal metabolite, *cis*-2-butene-1,4-dial, is likely to be an important intermediate in the conversion of furan to carbon dioxide and other excreted metabolites (Scheme 1). The urinary and fecal metabolites of furan have not been chemically characterized. However, HPLC analysis of urine from [^{14}C]furan-treated rats indicated the presence of several polar metabolites that were proposed to be GSH or mercapturic acid conjugates of furan metabolites (5).

In this report, we describe studies designed to characterize the urinary metabolites of furan. *cis*-2-Butene-1,4-dial reacts rapidly with GSH to form mono- and *bis*-GSH reaction products (1 and 2, respectively, Scheme 1) (10–12). Therefore, we developed stable isotope mass spectral methods to detect the GSH conjugates of *cis*-2-butene-1,4-dial and their subsequent metabolites (Scheme 1 and 2) in the urine of furan-treated rats. These studies are the first step in the development of markers which can be used to assess furan exposure and metabolism in humans.

Methods

Caution

cis-2-Butene-1,4-dial is toxic and mutagenic in cell systems. Furan is toxic and carcinogenic in laboratory animals. Both chemicals should be handled with proper safety equipment and precautions.

Furan and trifluoroacetic acid were purchased from Acros Organics (Pittsburgh, PA). [$^{13}C_4$] Furan, *cis*-2-butene-1,4-dial, and the mono- and bis-GSH conjugates of *cis*-2-butene-1,4-dial were prepared as previously described (11,12,14,16). Furan was distilled before use and its purity was confirmed by NMR. Corn oil was purchased from Sigma Chemical Co. (St. Louis, MO). *tris*-(Carboxyethyl)phosphine (TCEP) was purchased from Strem Chemicals (Newburyport, MA). All other chemicals were reagent grade.

2-{3-[S-(*N*-Acetylcysteinyl)]-1*H*-pyrrol-1-yl}pentanedioic acid and 2-{2-[S-(*N*-acetylcysteinyl)]-1H-pyrrol-1-yl}pentanedioic acid

N-Acetylcysteine (26 mg, 0.16 mmol) was added to a solution of *cis*-2-butene-1,4-dial (0.16 mmol) in 1 M sodium phosphate, pH 7.4 (total volume: 0.7 mL). Fifteen minutes later, glutamate (23 mg, 0.16 mmol) was added to the reaction. After 1 hr at room temperature, the reaction products were isolated by preparative HPLC. The Phenomenex Bondclone C18 column (250 × 10 mm, 10 micron) was eluted with a 15 min linear gradient from 20 mM NH_4OAc, pH 4.9, to 20 mM NH_4OAc, pH 4.9, containing 25% acetonitrile (flow rate: 3 mL/min). Absorbance was monitored at 222 and 254 nm. The reaction products eluted at 13.3 and 15.5 min.

2-{3-[*S*-(*N*-Acetylcysteinyl)]-1*H*-pyrrol-1-yl}pentanedioic acid (3)—^{1}H NMR (300 MHz, D_2O) δ: 6.78 (s, 1H, H2′), 6.66 (s, 1H, H5′), 6.08 (s, 1H, H4′), 4.33 (m, 1H, H2), 4.12 (m, 1H, Cys αCH), 3.0 (m, 1H, Cys β CH_a), 2.75 (m, 1H, Cys β CH_b), 2.17 (m, 1H, $H3_a$), 1.99 (m, 1H, $H3_b$), 1.91 (m, 2H, H4), 1.84 (s, 3H, N-CH_3). MS *m/z*: 359 [M+H].

2-{2-[S-(*N*-Acetylcysteinyl)]-1*H*-pyrrol-1-yl}pentanedioic acid—^{1}H NMR (300 MHz, D_2O) δ: 6.87 (s, 1H, H5′), 6.26 (s, 1H, H3′), 6.07 (s, 1H, H4′), 4.88 (m, 1H, H2), 4.09

(m, 1H, Cys αCH), 2.83 (m, 1H, Cys β CH_a), 2.68 (m, 1H, Cys β CH_b), 2.26 (m, 1H, $H3_a$), 2.04 (m, 1H, $H3_b$), 1.90 (m, 2H, H4), 1.84 (s, 3H, N-CH_3). MS *m/z*: 359 [M+H].

2-{3-[*S*-(Glutathionyl)]-1*H*-pyrrol-1-yl}pentanedioic acid (4)

GSH (65 mg, 0.2 mmol) and *cis*-2-butene-1,4-dial (0.2 mmol) were combined in 150 mM sodium phosphate, pH 7.4 (total volume: 7 mL). Glutamate (61 mg, 0.2 mmol) was added after 15 minutes. The desired product was isolated from a complex mixture by preparative HPLC. The HPLC column was eluted with a 19 min linear gradient from 100 mM ammonium acetate to 100 mM ammonium acetate containing 10% acetonitrile. ^{1}H NMR (300 MHz, D_2O) δ: 6.75 (s, 1H, H2′), 6.65 (s, 1H, H5′), 6.05 (s, 1H, H4′), 4.32 (m, 1H, H2), 4.20 (m, 1H, Cys αCH), 3.60 (t, 1H, Glu αCH), 3.5 (s, 2H, Gly CH_2), 3.0 (dd, 1H, Cys β CH_a), 2.68 (dd, 1H, Cys β CH_b), 2.30 (m, 2H, Glu γ CH_2), 1.90-2.25 (m, 6H, Glu β CH_2, H3, H4). MS *m/z*: 503 [M+H].

N-{4-Carboxy-4-[3-*S*-(*N*-acetylcysteinyl)-1*H*-pyrrol-1-yl]-1-oxobutyl}-L-cysteinylglycine (5)

N-Acetylcysteine (260 mg, 1.6 mmol) was combined with *cis*-2-butene-1,4-dial (1.6 mmol) in water (total volume: 6.8 mL). After overnight at room temperature, GSH (480 mg, 1.6 mmol) was added to the reaction. One and a half h later, the reaction mixture was purified by preparative HPLC. A Phenomenex Synergi 4μ Hydro-RP 80A column (250 × 10 mm, 4μ) was isocratically eluted with 20 mM NH_4OAc, pH 4.9, containing 4% acetonitrile (flow rate = 3 mL/min). Absorbance was monitored at 222 and 254 nm. The major reaction product eluted at 11.0 minutes: ^{1}H NMR (300 MHz, D_2O) δ: 6.89 (s, 1H, H2′), 6.81 (s, 1H, H5′), 6.23 (s, 1H, H4′), 4.51 (m, 1H, H4), 4.47 (m, 1H, Cys α CH), 4.21 (m, H, AcCys α CH), 3.72 (s, 2H, Gly CH_2), 3.15 (m, 1H, H- AcCys β CH_a), 2.93 (m, 1H, Cys β CH_a), 2.85 (m, 1H, Cys β CH_b), 2.82 (m, 1H, AcCys β CH_b), 2.41 (m, 1H, $H2_a$), 2.26 (m, 1H, $H2_b$), 2.24 (m, 1H, $H3_a$), 2.2 (m, 1H, $H3_b$), 1.82 (s, 3H, N-CH_3). MS m/z: 519 [M+H].

N-{4-Carboxy-4-[3-*S*-(*N*-acetylcysteinyl)-1H-pyrrol-1-yl]-1-oxobutyl}-L-cysteinylglycine disulfide

N-Acetylcysteine (25 mg, 0.15 mmol) and *cis*-2-butene-1,4-dial (0.15 mmol) were combined in 115 mM sodium phosphate, pH 7.4 (total volume: 2.6 mL). Oxidized GSH (47 mg, 0.077 mmol) was added after 30 minutes at room temperature. Thirty min later, the reaction product was purified by preparative HPLC. A Phenomenex Synergi 4μ Hydro-RP 80A column (250 × 10 mm, 4μ) was eluted with a 15 min linear gradient from 20 mM NH_4OAc to 20 mM NH_4OAc containing 15% acetonitrile. Fractions containing the product were concentrated under reduced pressure (95 mg, 60%). Compound **5** can be generated directly from this material by reacting it with TCEP. ^{1}H NMR (300 MHz, D_2O) δ: 6.71 (s, 1H, H2′), 6.62 (s, 1H, H5′), 6.03 (s, 1H, H4′), 4.52 (m, 1H, Cys α CH), 4.26 (m, 1H, H4), 3.98 (m, H, AcCys α CH), 3.55 (s, 2H, Gly CH_2), 3.05 (m, 1H, H- Cys β CH_a), 2.93 (m, 1H, AcCys β CH_a), 2.74 (m, 2H, Cys β CH_b, AcCys β CH_b), 2.30 (m, 1H, $H2_a$), 2.00 (m, 3H, $H2_b$, H3), 1.84 (s, 3H, N-CH_3). MS *m/z*: 1035 [M+H].

Furan Treatment of Rats

F344 male rats weighing 200–300 g were purchased from Charles River Laboratories (Kingston, NY). Groups of three rats were treated with 8 mg/kg [$^{12}C_4$]- or [$^{13}C_4$]furan in 5 ml/kg corn oil by gavage. A control group received only corn oil. Immediately after treatment, rats were transferred to individual metabolism cages. Urine was collected on dry ice for 24 hours. It was stored in glass tubes at −80 °C.

LC-MS Analysis of Urine

Urine was acidified with TFA to a final concentration of 2%, centrifuged to remove any particulate matter and filtered through a 0.45 μm syringe filter. The samples were stored at

−20° C in glass autosampler vials until analysis. In some cases, the urine was treated with TCEP to reduce any disulfide bonds (17,18).

Urine (8 μL) was eluted from an Agilent (Palo Alto, CA) Zorbax C18 capillary column (0.5 × 150 mm; 5 micron) with 50 mM ammonium formate, pH 2.8. After a 10 min isocratic period, the column was eluted with a 26 min linear gradient to 37.5 mM ammonium formate, pH 2.8 containing 12.5% acetonitrile. This, in turn, was followed by a 10 min gradient to 25 mM ammonium formate, pH 2.8, containing 25% acetonitrile (retention times: **1**, 24 min; **4**, 33 min; **2**, 35 min, **3**, 39 min, and **5**, 42 min). The column was washed with water containing 50% acetonitrile prior to returning to initial conditions. The HPLC was coupled to either an Agilent 1100 series LC/MSD Trap SL mass spectrometer or a ThermoFinnigan Quantum triple quadrapole mass spectrometer with an electrospray ionization source in positive ion mode. Initial analyses were performed with the mass spectrometer set to full scanning mode with a scan range of 100–750 m/z. Urine from individual animals treated with [$^{12}C_4$]- or [$^{13}C_4$]furan were analyzed separately as well as a 1:1 mixture. In some cases, the samples were treated with TCEP prior to MS analysis to reduce any disulfide bonds (12). The resulting data were searched for compounds that were present in the samples from furan-treated rats, absent in the samples from control rats and shifted by four amu in the urine from [$^{13}C_4$]furan-treated treated rats. The samples were then reanalyzed with the mass spectrometer set in the autoMS(n) mode to obtain fragmentation patterns for each of the metabolites.

The entire volume of urine from one furan-treated rat was treated with TFA as described above (total volume = ~4 mL), filtered and fractionated by HPLC. The urinary metabolites were eluted from a Phenomenex (Torrance, CA) Bondclone semi-prep scale column (250 × 10 mm, 10 micron) with 50 mM ammonium formate, pH 2.8. A 10 min isocratic period was followed by a 40 min linear gradient to 37.5 mM ammonium formation, pH 2.8, containing 12.5% acetonitrile and then a 10 min gradient to water containing 50% acetonitrile (flow rate = 4 mL/min). The UV absorbance was monitored at 254 nm. The fraction containing the monoGSH conjugate was used to obtain high resolution mass spectral information. This data was collected on a ThermoFinnigan Quantum triple quadrapole mass spectrometer with an electrospray ionization source in positive ion mode.

Results and Discussion

We have shown that *cis*-2-butene-1,4-dial reacts rapidly with GSH to form both mono- and *bis*-GSH reaction products in microsomes (1 and 2, respectively, Scheme 1) (11,12). Therefore, if *cis*-2-butene-1,4-dial is formed in vivo, we expected that it would be trapped by GSH. We also expected that these reaction products may serve as substrates for the enzymes involved in processing GSH conjugates to mercapturic acid derivatives. The mono-glutathione conjugate of *cis*-2-butene-1,4-dial (**1**) would ultimately be converted to 2-{3-[S-(*N*-acetylcysteinyl)]-1*H*-pyrrol-1-yl}pentanedioic acid (**3**), in the case of the 3-substituted pyrrole isomer (Scheme 2). Compound **3** might also be the product resulting from processing of both GSH moieties in the bis-GSH-*cis*-2-butene-1,4-dial-reaction product (**2**). Two intermediates in the conversion of **2** to **3** are 2-{3-[*S*-(glutathionyl)]-1*H*-pyrrol-1-yl}pentanedioic acid (**4**) or *N*-{4-carboxy-4-[3-*S*-(*N*-acetylcysteinyl)-1*H*-pyrrol-1-yl]-1-oxobutyl}-L-cysteinylglycine (**5**). These compounds (**3**–**5**) were prepared by adapting the procedure for preparing the *cis*-2-butene-1,4-dial-GSH reaction products (**1** and **2**) (11,12) and characterized by NMR and mass spectrometry. All these standards were then employed to develop chromatographic methods for their separation by capillary LC (Figure 1).

To determine the urinary metabolites of furan, groups of three F344 rats were treated with either [$^{12}C_4$]- or [$^{13}C_4$]furan (8 mg/kg, p.o.; in corn oil). A control group received vehicle alone. The animals were housed in metabolism cages for the collection of urine for 24 h. The

urine was acidified with trifluoroacetic acid with the precipitate removed by centrifugation. The samples were then subjected to C18 capillary LC/MS/MS analysis with the mass spectrometer operating in full scanning mode. Data for the GSH and mercapturic acid derivatives of *cis*-2-butene-1,4-dial were extracted from the total ion current.

The only predicted metabolite detected in the urine from treated rats was the mono-GSH conjugate of *cis*-2-butene-1,4-dial. A peak was observed for *m/z* 356 in the urine of furan treated animals that had the same retention time as the standard for the mono-GSH conjugates (Figure 2A). A similar peak was observed at *m/z* 360 in the urine from [$^{13}C_4$]furan-treated rats (Figure 2B). The fragmentation pattern for the urinary metabolite is identical to the synthetic standard and the masses are shifted by 4 a.m.u. in the metabolite derived from [$^{13}C_4$]furan (Figure 3). In addition, high resolution mass spectral analysis is consistent with the proposed structure (accurate mass for $C_{14}H_{18}N_3O_6S$ = 356.0916; mass of synthetic standard = 356.0894; mass of metabolite = 356.0904).

The observation of this metabolite in the urine of furan-treated rats indicates that *cis*-2-butene-1,4-dial is formed and trapped with GSH *in vivo*. The major reaction product formed between *cis*-2-butene-1,4-dial and GSH in vitro are the bis-GSH conjugates (11,12). Their absence does not mean that they are not formed *in vivo*. Given their size, they may be preferentially eliminated via the bile as has been observed for the GSH conjugates of structurally related 4-ipomeanol (19). We are investigating whether these compounds and their metabolites are presented in the feces of furan-treated rats.

At least 18 other furan-derived urinary metabolites were also detected (data not shown). Preliminary analysis of the mass spectral data indicates that they are conjugates and that the furan-derived portion of the molecule results from further metabolism of *cis*-2-butene-1,4-dial. We are currently isolating the individual metabolites for more in-depth chemical characterization.

In conclusion, the mono-GSH reaction product of *cis*-2-butene-1,4-dial was observed in urine of furan treated rats. The presence of this metabolite indicates that *cis*-2-butene-1,4-dial is formed in vivo. Preliminary data provide evidence that this reactive metabolite of furan also undergoes further biochemical transformation to other compounds. These metabolites will be explored for their suitability as markers for the uptake and metabolism of furan in human risk assessment studies.

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Abbreviations

GSH
: glutathione

TCEP
: *tris*-(2-carboxyethyl)phosphine

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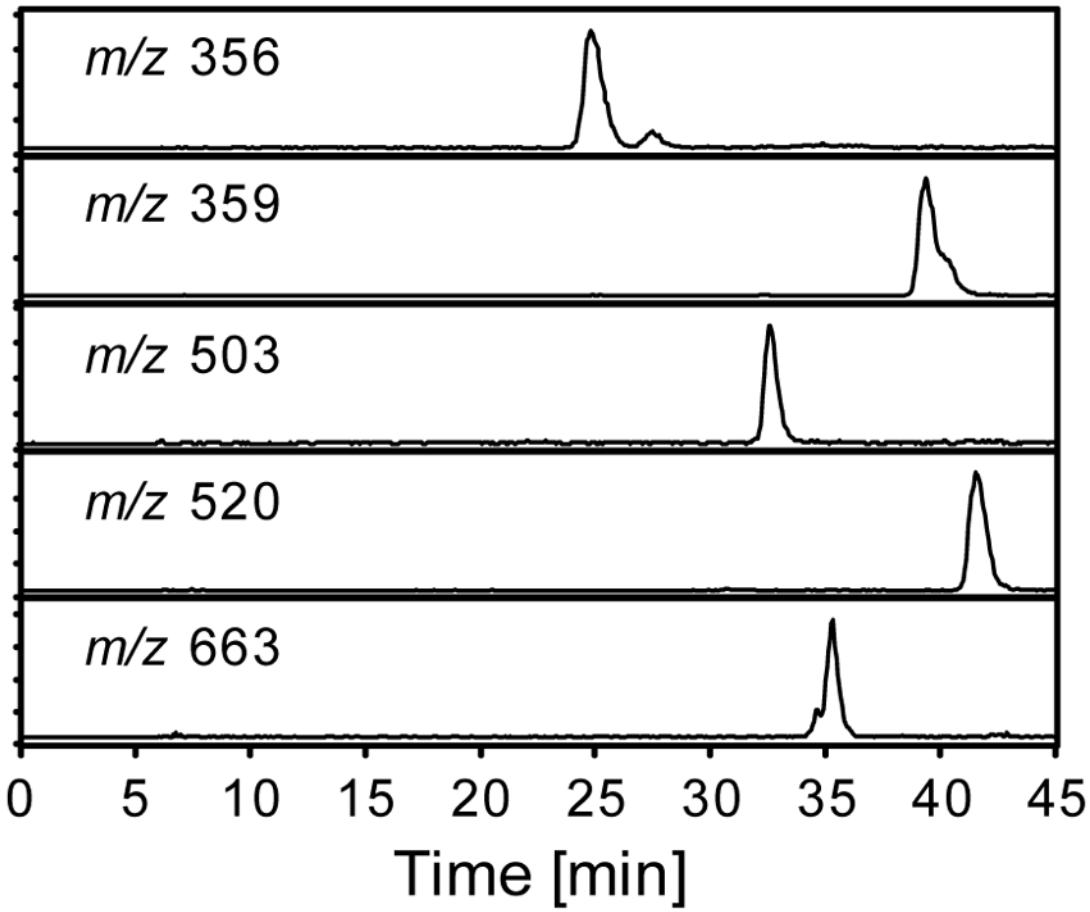


Figure 1.
Extracted chromatograms for the expected GSH reaction products and their metabolites. The molecular ions for the metabolites are as follows: **1**, m/z 356; **3**, m/z 359; **4**, m/z 503; **5**, m/z 520 and **2**, m/z 663.

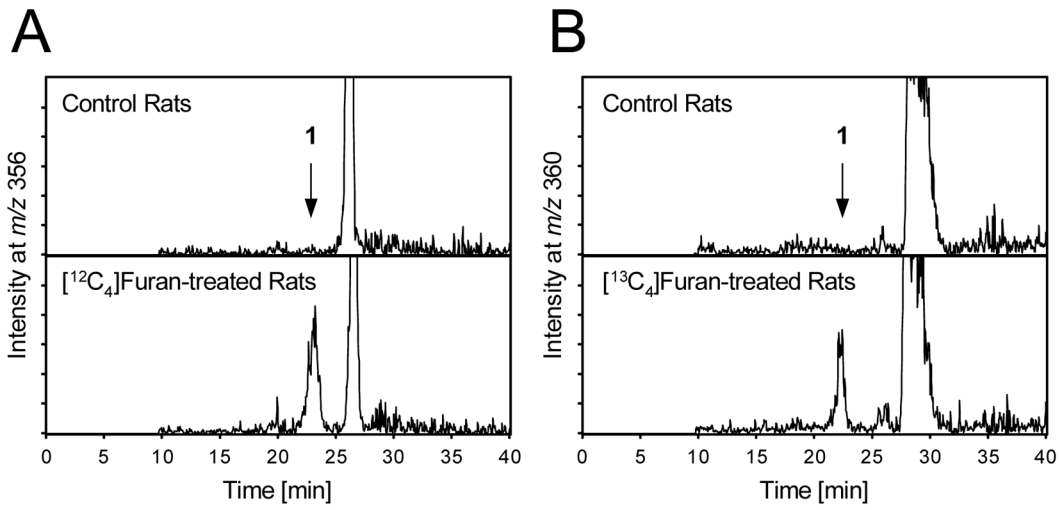


Figure 2.
Extracted mass chromatograms obtained at A) *m/z* 356 for urine from untreated or [$^{12}C_4$]furan-treated rats and B) *m/z* 360 for urine from untreated or [$^{13}C_4$]furan-treated rats.

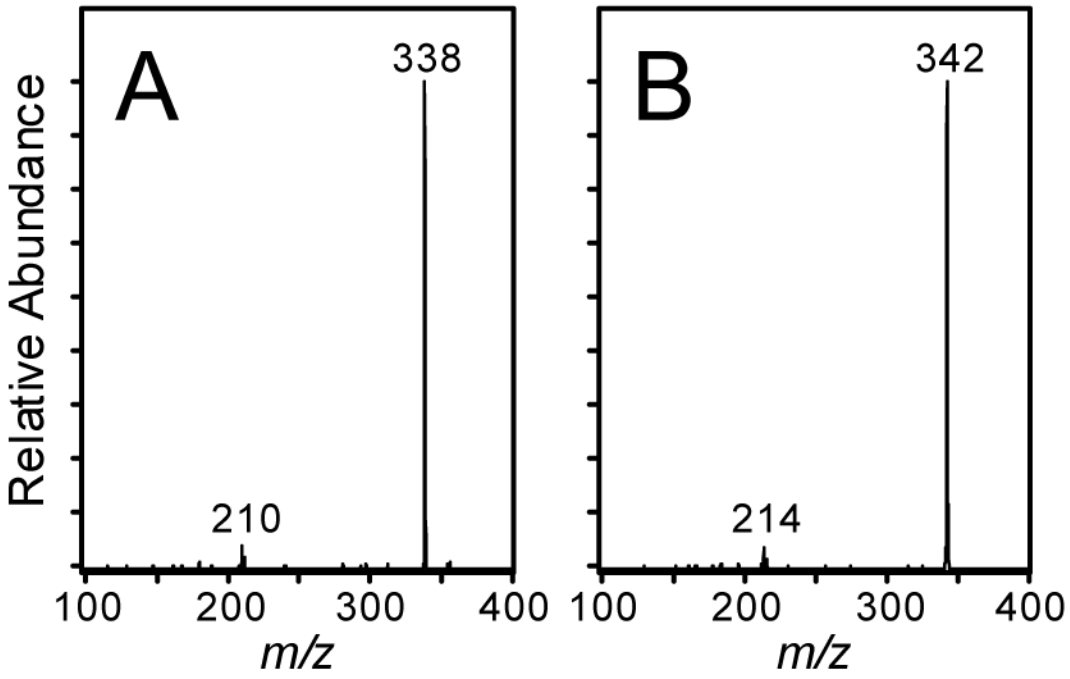
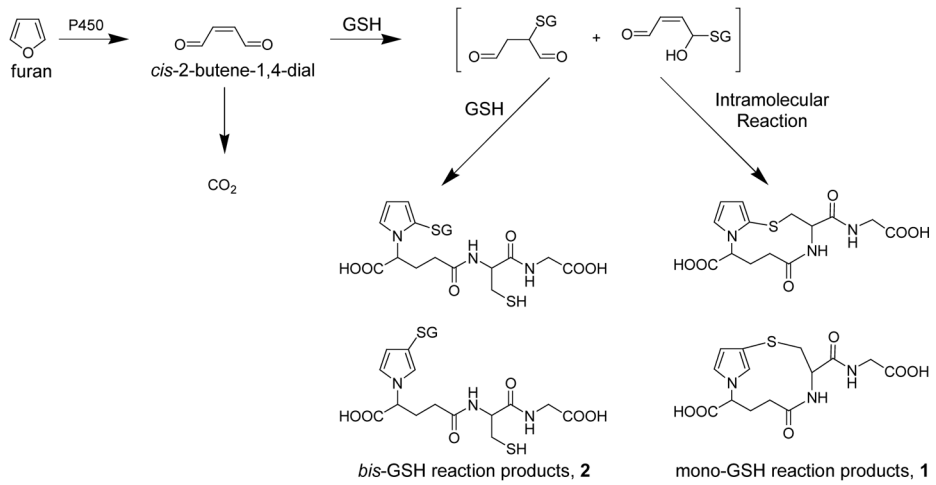
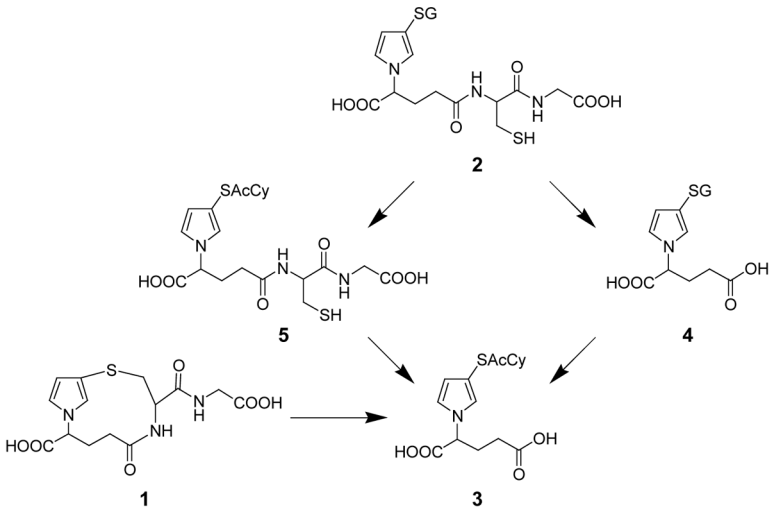


Figure 3.
Collision induced mass spectra of the monoGSH conjugate detected in urine from rats treated with A) [$^{12}C_4$]furan or B) [$^{13}C_4$]furan.



Scheme 1.
Proposed in vivo metabolic pathways for furan.



Scheme 2.

Further metabolism of the conjugates of *cis*-2-butene-1,4-dial. The 2-substituted isomer will generate structurally similar metabolites. GS, glutathione; AcCy, *N*-acetylcysteine.