GLUTATHIONE TRAPPING TO MEASURE MICROSOMAL OXIDATION OF FURAN TO CIS-2-BUTENE-1,4-DIAL

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ABSTRACT:

Furan is a liver carcinogen and toxicant. Furan is oxidized to the reactive dialdehyde, cis-2-butene-1,4-dial, by microsomal enzymes. This reactive metabolite readily reacts with glutathione nonenzymatically to form conjugates. A high-performance liquid chromatography-electrochemical method for the detection of cis-2-butene-1,4-dial-glutathione (GSH) conjugates in microsomal preparations was developed to measure the extent of furan metabolism to cis-2-butene-1,4-dial in vitro. Previously unobserved mono-GSH reaction products of cis-2-butene-1,4-dial were detected in addition to the already characterized bis-GSH conjugates. Chemical characterization of these compounds indicated that the α-amino group of glutathione had reacted with cis-2-butene-1,4-dial to form a thiol-substituted pyrrole adduct. The analytical method was used to estimate the extent of furan oxidation in rat liver microsomes from untreated or acetone-pretreated F344 rats as well as in human P450 2E1 Supersomes. Our results confirm that cytochrome P450 2E1 can catalyze the oxidation of furan to cis-2-butene-1,4-dial. However, the data are also consistent with the involvement of other P450 enzymes in the oxidation of furan in untreated animals. This assay will be a valuable tool to explore tissue and species differences in rates of furan oxidation.

Furan is a widely used industrial chemical that is also present in the environment (Capurro, 1973; Maga, 1979; International Agency for Research on Cancer, 1995). The human health effects of furan are unknown. It is both hepatotoxic and carcinogenic after oral administration in mice and rats, inducing cholangiocarcinomas and hepatocellular carcinomas (Maronpot et al., 1991; Elmore and Sirica, 1993; National Toxicology Program, 1993). Based on these results and the large potential for human exposure, furan has been listed as a possible human carcinogen by the National Toxicology Program and the International Agency for Research on Cancer (International Agency for Research on Cancer, 1995, 1997).

Based on the results of previous studies, the formation of this metabolite was monitored by trapping with semicarbazide or [3H]GSH as the bis-semicarbazone or the bis-GSH conjugates, respectively (Chen et al., 1995, 1997). The initial oxidation product of furan is cis-2-butene-1,4-dial (Fig. 1). This compound is reactive and difficult to isolate and characterize directly. In previous studies, the formation of this metabolite was monitored by trapping with semicarbazide or [3H]GSH as the bis-semicarbazone or the bis-GSH conjugates, respectively (Chen et al., 1995, 1997).

We were interested in determining the kinetic parameters of the oxidation of furan by microsomal preparations from various species and tissues. These studies required the development of an assay that would provide a quantitative estimate of cis-2-butene-1,4-dial concentrations. Given the reactive nature of this metabolite, such an assay requires the presence of a trapping agent to prevent protein binding. The use of semicarbazide was not explored since it is a potential inhibitor of the cytochrome P450-mediated oxidation of furan. GSH was chosen as the trapping agent since it protects against the majority of cytochrome P450-catalyzed protein binding of [14C]furan (Parmar and Burka, 1993) and will not inhibit cytochrome P450. Trapping reactive metabolites with GSH or other sulfhydryl reagents has been used as a method to estimate the extent of metabolic activation for a number of drugs and environmental compounds (Tang et al., 1999; Smith et al., 2003; Alvarez-Diez and Zheng, 2004; Baer et al., 2005; Gan et al., 2005). In this report, we describe the development of an HPLC-electrochemical (HPLC-EC) detection method for the detec-
tion of cis-2-buten-1,4-dial-GSH conjugates and its application to determine the extent of the cytochrome P450-catalyzed oxidation of furan in microsomal preparations.

Materials and Methods

Solutions of cis-2-buten-1,4-dial were prepared and quantified as reported previously (Peterson et al., 2000; Byrns et al., 2004). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, NADP⁺, GSH, and p-nitrophenol were obtained from Sigma-Aldrich (St. Louis, MO). Furan was obtained from Acros Organics (Fairlawn, NJ) and was distilled before storage at −20°C. Tris(2-carboxyethyl)phosphine (TCEP) was purchased from Strem Chemicals (Newburyport, MA). All other chemicals used were reagent grade obtained from commercial sources. 1H NMR spectra were obtained with either a Varian Inova-300 or 500 NMR spectrometer in CDCl3 and are reported in ppm relative to an external standard.

Preparation of Rat Liver Microsomes. Male F344 rats (200–300 g) were purchased from Charles River Laboratories (Kingston, NY). In some cases, the rats received 1% acetone in their drinking water for 1 week before sacrifice (Kedderis et al., 1993). Liver microsomes were isolated as described previously (Guengerich, 1982).

Identification of Mono-GSH Conjugates of cis-2-Buten-1,4-Dial. cis-2-Buten-1,4-dial (0.42 mg; 5.0 μmol) and GSH (7.7 mg; 25 μmol) were combined in 100 mM potassium phosphate buffer, pH 7.4 (total volume 0.5 ml). After 30 min at room temperature, TCEP (7.2 mg; 25 μmol) was added, and the reaction was continued for an additional 30 min. cis-2-Buten-1,4-dial-GSH conjugates were purified by HPLC with UV detection on a Synergi 80A column (Phenomenex, Torrance, CA; 46.5 × 250 mm, 4 μm) using solvents A (100 mM ammonium acetate) and B (95% acetonitrile) at a flow of 1 ml/min. The mixture was eluted with a linear gradient from 100% A to 90% A/10% B over 15 min. The mono-GSH conjugate eluted at 6 min. [N-[4-Carbonyl-4-(2-mercapto-1H-pyrrol-1-yl)-1-oxobutyl]-l-cysteinylglycine cyclic sulfide, isomer 1: 1H NMR (300 MHz, D2O) δ 6.85 (bs, 1H, H-5), 6.58 (d, 1H, H-3), 6.09 (d, 1H, H-6), 4.60 (m, 1H, Glu α-CH), 4.24 (d, 1H, Cys α-CH), 3.62 (s, 2H, Gly CH2), 2.74–2.86 (m, 2H, Cys β-CH), 2.1–2.3 (m, 4H, Glu γ-CH2 and β-CH2), N-[4-Carbonyl-4-(3-mercapto-1H-pyrrol-1-yl)-1-oxobutyl]-l-cysteinylglycine cyclic sulfide, isomer 2: 1H NMR (300 MHz, D2O) δ 6.73 (bs, 1H, H-5), 6.70 (bs, 1H, H-2), 6.26 (d, 1H, H-4), 4.60 (m, 1H, Glu α-CH), 4.16 (d, 1H, Cys α-CH), 3.62 (s, 2H, Gly CH2), 3.30 (m, 1H, Cys β-CH), 2.90 (m, 1H, Cys β-CH2), 2.1–2.3 (m, 4H, Glu γ-CH2 and β-CH2).

Microsomal Metabolism of Furan. Furan (0–4 mM) was incubated in the presence of untreated or acetone-pretreated rat liver microsomes (250 or 50 μg/ml, respectively) or human P450 2E1 Supersomes (15 μg/ml) containing 100 mM potassium phosphate buffer, pH 7.4, 25 mM glucose-6-phosphate, 2 units/ml glucose-6-phosphate dehydrogenase, 4 mM NADP⁺, 3 mM MgCl2, 1 mM EDTA, and 8 mM GSH for 10–60 min at 37°C in sealed tubes (final volume 250–500 μl). Incubations were started by the addition of furan as an aqueous solution. This solution was prepared by initially dissolving furan (0.5 M) in acetonitrile. This concentrated solution was then diluted with water to obtain the final solution added to the microsomal incubations. The final concentration of acetonitrile in the microsomal incubations never exceeded 0.4%. Each reaction was performed in triplicate. Controls were performed in the absence of NADPH, furan, or GSH. In some cases, the incubations were performed in the presence of p-nitrophenol (50 or 100 μM). The reactions were terminated by adding 0.3 N NaOH and 0.3 N ZnSO4 (25–50 μl each). The precipitate was removed by centrifugation, and the supernatant was filtered through a 0.45-μm 0.4-mm nylon syringe filter (Millex-HN; Millipore Corporation, Billerica, MA). The filtrate (84 μl) was combined with 40 mM TCEP (12 μl), and the resulting solution was analyzed by HPLC with EC detection (injection volume 75 μl). The HPLC system consisted of an ESA (Chelmsford, MA) CouArray electrochemical detector equipped with two electrochemical cells with four channels each. ESA 582 HPLC pumps, and an ESA 542 autosampler. The system was operated through the CouArray for Windows software, version 1.04, from ESA. The incubation mixtures were analyzed on a Bondclone C18 column (Phenomenex, Torrance, CA; 300 × 3.9 mm; 10 μm) using solvents C (100 mM potassium phosphate buffer, pH 2) and D (acetonitrile containing 5% water). They were separated with a linear gradient from 97% C/3% D to 75% C/25% D over 25 min at a flow rate of 1 ml/min. The electrochemical detector’s channel 1 was set at −700 mV, and channel 2 was set at +675 mV. The −700-mV potential of the first channel was used to ensure all analytes were fully reduced before detection by oxidation at +675 mV.

Calibration curves for cis-2-buten-1,4-dial-GSH conjugates were prepared by incubating cis-2-buten-1,4-dial (0–100 μM) in the presence of untreated or acetone-pretreated rat liver microsomes (250 or 50 μg/ml, respectively) or human P450 2E1 Supersomes (15 μg/ml) containing 100 mM potassium phosphate buffer, pH 7.4, 25 mM glucose-6-phosphate, 2 units/ml glucose-6-phosphate dehydrogenase, 4 mM NADP⁺, 3 mM MgCl2, 1 mM EDTA, and 8 mM GSH for 60 min at 37°C (total volume 250 μl). The incubations were stopped by the addition of 0.3 N NaOH and 0.3 N ZnSO4 (25 μl each).
FIG. 2. Electrochemical chromatograms of a reaction mixture of cis-2-butene-1,4-dial and GSH (A), a microsomal incubation mixture of 1.5 mM furan in the presence of required cofactors (B), a microsomal incubation in the absence of furan (C), and a microsomal incubation of 1.5 mM furan in the absence of NADPH (D). The microsomal incubations were performed with untreated rat liver microsomes (250 μg protein/ml) in the presence of 8 mM GSH and required cofactors for 10 min at 37°C. TCEP was added before the analysis to reduce any disulfide bonds (Burns et al., 1991; Krijt et al., 2001).

Remião et al., 2000). The HPLC-EC detection method was developed with a solution of cis-2-butene-1,4-dial in the presence of excess GSH; cis-2-butene-1,4-dial reacts readily with GSH to form GSH reaction products (Chen et al., 1997). This reaction is complete within 15 min (data not shown). HPLC analysis of this reaction mixture indicated the presence of two major peaks corresponding to reaction products (Fig. 2A).

To confirm the identity of the electrochemically active peaks as GSH reaction products, the reaction mixtures were analyzed by LC-MS/MS analysis. The second peak (12.5 min) contains both the 2- and 3-substituted bis-GSH conjugates (Fig. 1). Consistently, the molecular ion of the compounds contained in this peak was m/z 663 (Fig. 3A), and the daughter ion spectrum was consistent with the formation of the previously characterized bis-GSH conjugates of cis-2-butene-1,4-dial (Figs. 1 and 3A) (Chen et al., 1997). The earlier eluting reaction product (11 min) produced a molecular ion at m/z 356. The daughter ion spectrum contains an ion at m/z 338 which results from the loss of water (Fig. 3A). This molecular ion is consistent with the formation of a mono-GSH conjugate (Fig. 1). The absence of significant fragmentation patterns indicated that both the amino and the sulfhydryl groups of GSH are involved in conjugate formation. 1H NMR data support the conclusion that the α-amino group of GSH is involved in pyrrole ring formation. The patterns of the aromatic protons indicate that the thiol group of GSH is attached to either the 2- or 3-position of the pyrrole ring (Chen et al., 1997). Integration of these aromatic protons is consistent with an approximately 1:1 mixture of the 2- and 3-substituted cyclic mono-GSH reaction product (Fig. 1).

Storage of cis-2-butene-1,4-dial-GSH solutions led to the formation of a complicated mixture (data not shown). Although the mono-GSH reaction products seem to be stable, the bis-GSH reaction products readily formed mixed disulfides with themselves as well as with GSH (Chen et al., 1997). The addition of 4 mM TCEP, which reduces
disulfide bonds (Burns et al., 1991; Krijt et al., 2001), removed this complexity in the HPLC traces. All subsequent analyses were performed after the addition of TCEP to maintain the reduced state of any free thiol groups. Under these reductive conditions, the cis-2-butene-1,4-dial-GSH products were stable for several days as judged by HPLC analysis.

The signal for the cis-2-butene-1,4-dial-GSH conjugates was maximal at an electrode potential of +675 mV. Quantification of the cis-2-butene-1,4-dial-GSH conjugates was achieved through the use of calibration curves for the GSH conjugates (Fig. 4). The calibration solutions were prepared by reacting increasing amounts of cis-2-butene-1,4-dial with an excess of GSH in the presence or absence of microsomal protein. The limits of detection for the GSH-conjugates were approximately 25 pmol on column. A linear correlation was observed between the cis-2-butene-1,4-dial concentration and each of the HPLC peaks corresponding to the various GSH-cis-2-butene-1,4-dial reaction products, but we found it convenient to sum all of these peaks for our studies below. Figure 4 displays the calibration curve for cis-2-butene-1,4-dial using the total sum of the peak areas ($r^2 = 0.99$; Fig. 4). The variation between multiple samples prepared at the same concentration of cis-2-butene-1,4-dial and analyzed within 3 days of preparation was 2 to 10%. The variation increased when the samples were stored frozen for more than 1 week. The reason for this variation is unknown. To reduce error in the analyses, all samples were analyzed within 3 days of preparation.

The levels of the GSH-reaction products were slightly lower when the analysis was repeated in the presence of microsomal proteins but it remained linear (Fig. 4). The lower levels were not unexpected considering the reactive nature of cis-2-butene-1,4-dial; GSH protects against most but not all protein binding of [14C]furan to microsomal proteins (Parmar and Burka, 1993). The presence of the complete microsomal system did not seem to alter the relative ratio of the mono- versus bis-GSH-reaction products. The calibration curves were conducted in the presence of the same concentration of microsomal protein as the furan-containing incubations. These calibration curves were run each day since there was some variation in the electrochemical response.

The HPLC-EC analytical method was applied to estimate the extent of microsomal oxidation of furan to cis-2-butene-1,4-dial. The formation of the GSH conjugates required the presence of furan, GSH and
NADPH (Fig. 2, B–D). LC-MS/MS analysis confirmed that the conjugates detected by HPLC-EC were the same conjugates observed in reactions of GSH and cis-2-butene-1,4-dial (Fig. 3B).

The kinetics of furan oxidation to cis-2-butene-1,4-dial was measured in rat liver microsomes from both untreated and acetone-pretreated rats as well as human P450 2E1 Supersomes (Table 1). Reaction times and protein levels were adjusted so that less than 5% of furan had been converted to the GSH-conjugates. This allowed for more accurate determination of the kinetic parameters. Acetone pretreatment of the rats lowered the $K_m$ but increased the $V_{max}$ for furan oxidation in liver microsomes. The increased rate of oxidation is consistent with previous reports that acetone increases cytochrome P450 2E1 activity as well as furan metabolism in hepatocytes (Kedderis et al., 1993). The unexpected change in $K_m$ may indicate that there are other P450s contributing to the oxidation of furan in the uninduced microsomes. Consistent with this hypothesis, furan oxidation was less sensitive to inhibition by the P450 2E1 substrate, p-nitrophenol, in the uninduced microsomes (Fig. 5). Human P450 2E1 Supersomes containing cytochrome $b_5$ also catalyzed the oxidation of furan, with a $K_m$ higher than that observed with rat liver microsomes (Table 1). p-Nitrophenol inhibited the formation of cis-2-butene-1,4-dial-GSH conjugates to a similar extent in the human P450 2E1 Supersomes and the microsomes from acetone-pretreated rats (Fig. 5).

Previously, Kedderis and coworkers reported evidence that P450 2E1 was responsible for the metabolism of furan in rat hepatocytes (Kedderis et al., 1993). Furan metabolism was determined by measuring the disappearance of furan, with a $K_m$ of 0.4 mM in rat hepatocytes. Subsequent studies with human hepatocytes yielded a $K_m$ in the range of 2.1–3.3 mM (Kedderis and Held, 1996).

The $K_m$ in our microsomal reactions was at least an order of magnitude higher than that observed in the hepatocytes experiments. The reason for this discrepancy is unknown. The methods of analysis are different in the two studies; the kinetic parameters in the hepatocyte studies were obtained by measuring the disappearance of furan, whereas the kinetic parameters in our experiments were determined by measuring product formation. We believe that the oxidation of furan to cis-2-butene-1,4-dial is the overall rate-determining step in the formation of the GSH conjugates since the reaction with GSH is very rapid and is not thought to involve an enzymatically mediated pathway. In the hepatocyte studies, the observed kinetics is a composite of a larger variety of rate-determining steps. The actual concentration at the site of the enzymes is unknown.

In summary, we have developed an assay for the quantification of cis-2-butene-1,4-dial-GSH conjugates formed in metabolic reactions. This led to the identification of a previously uncharacterized GSH-reaction product, the mono-GSH conjugates. Preliminary results with rat liver microsomes indicate that cytochrome P450 2E1 is a catalyst for the oxidation of furan to cis-2-butene-1,4-dial but that other P450s may also be involved in the metabolic activation of this compound. This assay will be used to investigate the ability of other P450 enzymes to carry out this reaction as well as explore tissue and species differences in furan oxidation.

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References


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