DMD Fast Forward. Published on July 8, 2005 as doi:10.1124/dmd.105.004432

DMD #4432

Glutathione trapping to measure microsomal oxidation of furan to *cis*-2-butene-1,4-dial

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Running title: Glutathione trapping of *cis*-2-butene-1,4-dial

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Number of text pages: 10

Number of table: 1

Number of figures: 5

Number of references: 28

Number of words in abstract: 180

Number of words in introduction: 483

Number of words in discussion: 1313

Non-standard abbreviations: EC, electrochemical detection; GSH, glutathione; NP, *p*-nitrophenol; TCEP, *tris*-(2-carboxyethyl)phosphine.

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. An HPLC-electrochemical method for the detection of *cis*-2-butene-1,4-dial-GSH conjugates in microsomal preparations was developed in order 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 employed 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 is 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; International Agency for Research on Cancer, 1995; Maga, 1979). The human health effects of furan are unknown. It is both hepatoxic and carcinogenic after oral administration in mice and rats, inducing cholangiocarcinomas and hepatocellular carcinomas (Elmore and Sirica, 1993; Maronpot et al., 1991; 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; National Toxicology Program, 2000).

Furan toxicity requires metabolism. Furan is transformed into a protein binding intermediate via a cytochrome P450 dependent process both *in vivo* and *in vitro* (Burka et al., 1991; Parmar and Burka, 1993). The reactive metabolite is efficiently trapped with glutathione (GSH), reducing protein binding by greater than 85% (Parmar and Burka, 1993). Furan depletes GSH and reduces cell viability at biologically relevant doses in freshly isolated hepatocytes (Carfagna et al., 1993). Furan also depletes ATP in isolated hepatocytes and uncouples oxidative phosphorylation both *in vitro* and *in vivo* (Mugford et al., 1997). These effects are inhibited by cytochrome P-450 inhibitors such as 1-phenylimidazole and induced by acetone pretreatment (induction of cytochrome P450 2E1), paralleling the effects of inhibitors and inducers of furan metabolism (Kedderis et al., 1993).

The initial oxidation product of furan is *cis*-2-butene-1,4-dial (Figure 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 [^{3}H]GSH as the *bis*-semicarbazone or the *bis*-GSH conjugates, respectively (Chen et al., 1995; Chen et al., 1997).

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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 [^{14}C]furan (Parmar and Burka, 1993) and will not inhibit cytochrome P450. Trapping reactive metabolites with GSH or other sulfhydryl reagents has been employed as a method to estimate the extent of metabolic activation for a number of drugs and environmental compounds (Alvarez-Diez and Zheng, 2004; Baer et al., 2005; Gan et al., 2005; Smith et al., 2003; Tang et al., 1999). In this report, we describe the development of an HPLC-EC method for the detection of *cis*-2-butene-1,4-dial-GSH conjugates and its application to determine the extent of the cytochrome P450 catalyzed oxidation of furan in microsomal preparations.

Methods

Solutions of *cis*-2-butene-1,4-dial were prepared and quantified as previously reported (Byrns et al., 2004; Peterson et al., 2000). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, GSH, and p-nitrophenol were obtained from Sigma Chemical Co. (St. Louis, MO). Furan was obtained from Acros Organics (New Jersey) and was distilled prior to storage at –20°C. tris-(2-Carboxyethyl)phosphine (TCEP) was purchased from Strem Chemicals (Newburyport, MA). Human CYP2E1 Supersomes containing P450 reductase and cytochrome b_5 were obtained from BD Biosciences (Woburn, MA). All other chemicals used were reagent grade obtained from commercial sources. ^{1}H NMR spectra were obtained with either a Varian

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Inova-300 or 500 NMR spectrometer in $CDCl_3$ 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 one week prior to sacrifice (Kedderis *et al.*, 1993). Liver microsomes were isolated as previously described (Guengerich, 1982).

Identification of MonoGSH Conjugates of cis-2-butene-1,4-dial. *cis*-2-Butene-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-Butene-1,4-dial-GSH conjugates were purified by HPLC with UV detection on a Synergi HPLC column (Phenomenex, Torrance, CA; 4.6 x 250 mm, 4 micron) 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-Carboxy-4-(2-mercapto-1*H*-pyrrol-1-yl)-1-oxobutyl]-L-cysteinylglycine cyclic sulfide, isomer 1: ^{1}H NMR (300 MHz, D_2O) δ: 6.85 (bs, 1H, H-5), 6.58 (d, 1H, H-3), 6.09 (d, 1H, H-4), 4.60 (m, 1H, Glu α-CH), 4.24 (d, 1H, Cys α-CH), 3.62 (s, 2H, Gly CH_2), 2.74-2.86 (m, 2H, Cys β-CH_2), 2.1-2.3 (m, 4H, Glu γ-CH_2 and β-CH_2). *N*-[4-Carboxy-4-(3-mercapto-1*H*-pyrrol-1-yl)-1-oxobutyl]-L-cysteinylglycine cyclic sulfide, isomer 2: ^{1}H NMR (300 MHz, D_2O) 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 CH_2), 3.30 (m, 1H, Cys β-CHa), 2.90 (m, 1H, Cys β-CHb), 2.1-2.3 (m, 4H, Glu γ-CH_2 and β-CH_2).

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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 $MgCl_2$, 1 mM EDTA, and 8 mM GSH for 10-60 min at 37 ^oC 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 0.3N $Ba(OH)_2$ and 0.3N $ZnSO_4$ (25-50 μL each). The precipitate was removed by centrifugation and the supernatant was filtered through a 0.45 micron 0.4 mm nylon syringe filter (Millex-HN; Millipore, Bedford, 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) CoulArray 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 CoulArray for Windows software, v1.04 from ESA. The incubation mixtures were analyzed on a Bondclone C18 column (Phenomenex, Torrance, CA; 300 mm x 3.9 mm, 10 micron) 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 one was set at -700 mV and channel two was set at +675 mV. The -700 mV potential of the first channel was employed to ensure all analytes were fully reduced prior to detection by oxidation at +675 mV.

Calibration curves for *cis*-2-butene-1,4-dial-GSH conjugates were prepared by incubating *cis*-2-butene-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 $MgCl_2$, 1 mM EDTA, and 8 mM GSH for 60 min at 37 ^oC (total volume = 250 μL). The incubations were stopped by the addition of 0.3 N $Ba(OH)_2$ and 0.3 N $ZnSO_4$ (25 μL each). TCEP was added prior to analysis as described above. The sum of the peak areas for the mono- and *bis*-*cis*-2-butene-1,4-dial-GSH reaction products were plotted against the concentration of *cis*-2-butene-1,4-dial added to the reaction mixture.

In some microsomal incubations, there was a background peak that interfered with the signal for the mono-GSH conjugates. In these cases, we used only the *bis*-GSH conjugates to quantitate metabolism since the mono and *bis*-GSH conjugates were always formed in the same ratio, independent of *cis*-2-butene-1,4-dial or furan concentration.

Data Analysis. Kinetic parameters (K_m and V_{max}) were determined by curve fitting and nonlinear regression using SigmaPlot 2001 Enzyme Kinetics Module 1.1 **(**SPSS, Inc, Chicago, IL**)**. The values are the average ± S.D. from 2 experiments (n = 6 for each concentration).

LC-MS Analysis. The *cis*-2-butene-1,4-dial-GSH reaction mixture and 60 min microsomal incubation mixtures of 2 mM furan were analyzed by LC-MS. Analyses were performed with an Agilent Zorbax C18 capillary column (0.5 x 150 mm; 5 micron) linked to an

Agilent (Palo Alto, CA) 1100 series LC/MSD Trap SL mass spectrometer in positive ion mode. Full-scan LC/MS was performed with a scan range of *m/z* 150-1500, with MS/MS performed on the most abundant ion. The elution buffers were E (0.06% TFA in water) and F (acetonitrile containing 5% water). The mixtures were separated with a linear gradient from 97%E/3%F to 50% E/50% F over 25 min at a flow rate of 0.2 mL/min. The first five minutes were diverted to waste to reduce the amount of salt entering the mass spectrometer.

Results and Discussion

Initial attempts to measure the levels of GSH conjugate formation with [^{3}H]GSH were complicated by high radioactive background from the presence of excess [^{3}H]GSH (Chen *et al.*, 1997). Since radiolabeled furan was not available, non-radioactive methods were considered. We decided to explore HPLC with electrochemical (EC) detection since it has been used as a method for quantitation of GSH and other thiol containing compounds (Manna et al., 1999; Melnyk et al., 1999; 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 (Figure 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 (Figure 1). Consistently, the molecular ion of the compounds contained in this peak was *m/z* 663 (Figure 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 (Figures 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 (Figure 3A). This molecular ion is consistent with the formation of a mono-GSH conjugate (Figure 1). The absence of significant fragmentation patterns indicated that both the amino and the sulfhydryl groups of GSH are involved in conjugate formation. ^{1}H 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 (Figure 1).

Storage of *cis*-2-butene-1,4-dial-GSH solutions led to the formation of a complicated mixture (data not shown). While the mono-GSH reaction products appear 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 following 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 (Figure 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 display the calibration curve for *cis*-2-butene-1,4-dial using the total sum of the peak areas ($r^2 = 0.99$; Figure 4). The variation between multiple samples prepared at the same concentration of *cis*-2-butene-1,4-dial and analyzed within three days of preparation was 2-10%. 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 three 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 (Figure 4). The lower levels not unexpected considering the reactive nature of *cis*-2-butene-1,4-dial; GSH protects against most but not all protein binding of [^{14}C]furan to microsomal proteins (Parmar and Burka, 1993). The presence of the complete microsomal system did not appear to alter the relative ratio of the mono- versus *bis*-GSH-reaction products. The calibration curves were conducted in the presence of 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 (Figure 2B-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 (Figure 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 Km but increased the Vmax 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 Km 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 (Figure 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 (Figure 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. These studies indicated that the disappearance of furan was a single saturable process with a K_m of 0.4 μM in rat hepatocytes. Subsequent studies with human hepatocytes yielded a K_m in the range of 2.1-3.3 μM (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 quite 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 lead 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 employed to investigate the ability of other P450 enzymes to carry out this reaction as well as explore tissue and species differences in furan oxidation.

Acknowledgements

We thank Jacqueline Chan for her assistance in the chemical characterization of the *cis*-2-butene-1,4-dial-mono-GSH reaction product.

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Footnotes:

This research was funded by ES-10577 from the National Institutes of Health. An instrument grant from the Minnesota Medical Foundation was used to purchase the electrochemical detector.

Portions of this research were presented at the 2004 Fall National Meeting of the American Chemical Society in Philadelphia, PA as well as at the 7th International Meeting for the International Society for the Study of Xenobiotics in Vancouver, British Columbia.

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Figure Legends:

Figure 1: Metabolism of furan to *cis*-2-butene-1,4-dial and the subsequent reaction of *cis*-2-butene-1,4-dial with GSH to form mono- and *bis*-GSH reaction products.

Figure 2: Electrochemical chromatograms of A) a reaction mixture of *cis*-2-butene-1,4-dial and GSH; B) a microsomal incubation mixture of 1.5 mM furan in the presence of required cofactors; C) a microsomal incubation in the absence of furan and D) a microsomal incubation of 1.5 mM furan in the absence of NADPH. 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. tris(Carboxyethyl)phosphine (TCEP) was added prior to the analysis to reduce any disulfide bonds (Burns *et al.*, 1991; Krijt *et al.*, 2001).

Figure 3: Mass chromatograms and daughter ion spectra of the *cis*-2-butene-1,4-dial-GSH conjugates observed in A) reaction mixture of *cis*-2-butene-1,4-dial with GSH or B) microsomal incubations of 2 mM furan with rat liver microsomes in the presence of the required P450 cofactors and GSH.

Figure 4: Calibration curves for *cis*-2-butene-1,4-dial-GSH conjugates generated from *cis*-2-butene-1,4-dial (0-1 mM) and 8 mM GSH in the absence (square) and presence (diamond) of rat liver microsomal proteins (1 mg protein/mL) containing a NADPH regenerating system. Data are average values from three individual samples. The error bars represent standard deviation.

Figure 5: Inhibition of *cis*-2-butene-1,4-dial-glutathione conjugate formation in rat liver microsomes (RLM) and supersomes expressing human P450 2E1 by p-nitrophenol (NP). NP (0-100 μM) was added to incubations of furan (25 μM) with human 2E1 supersomes (15 μg protein/mL), or liver microsomes from either untreated (250 μg protein/mL) or acetone pretreated F-344 rats (50 μg protein/mL) in the presence of a NADPH regenerating system. The extent of furan metabolism was determined by EC-HPLC analysis.

DMD Fast Forward. Published on July 8, 2005 as DOI: 10.1124/dmd.105.004432
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Table 1. Kinetic parameters for the oxidation of furan to *cis*-2-butene-1,4-dial in GSH fortified microsomal preparations[a]

Microsomal preparation	K_m (μM)	V_{max} (nmol/min/mg protein)	V_{max}/K_m
Untreated rat liver microsomes	37.6 ± 4.3	2.5 ± 0.1	0.066
Acetone-pretreated rat liver microsomes	18.5 ± 6.8	5.9 ± 0.5	0.32
Human P450 2E1 supersomes	65.1 ± 16.4	15.4 ± 1.1[b]	0.24

[a]Furan (0, 10, 50, 100, 250 or 500 μM) was incubated in the presence of rat liver microsomes (untreated: 250 μg protein/mL; acetone pre-treated: 50 μg protein/mL) or human P450 2E1 supersomes (15 μg protein/mL) in the presence of a NADPH regenerating system and 8 mM GSH for 10 min. The values are the average ± S.D. from 2 experiments (n = 6 for each concentration).

[b]26 pmol/min/pmol P450.

Figure 1

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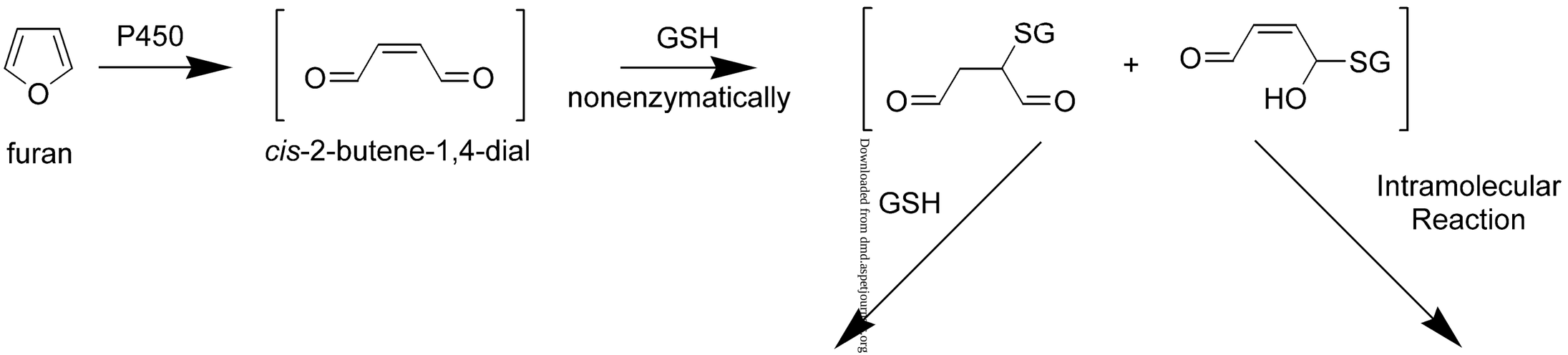


Figure 2

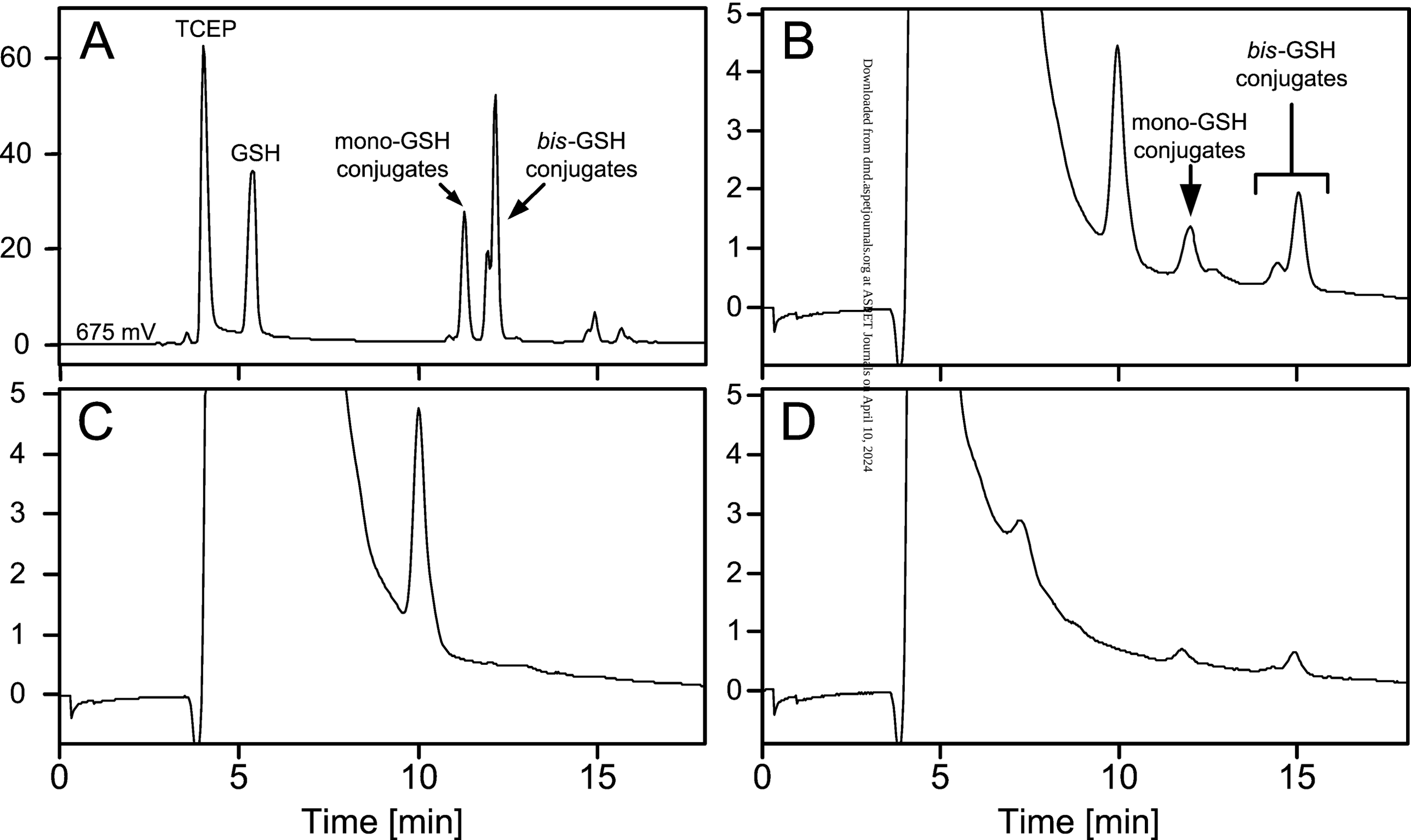
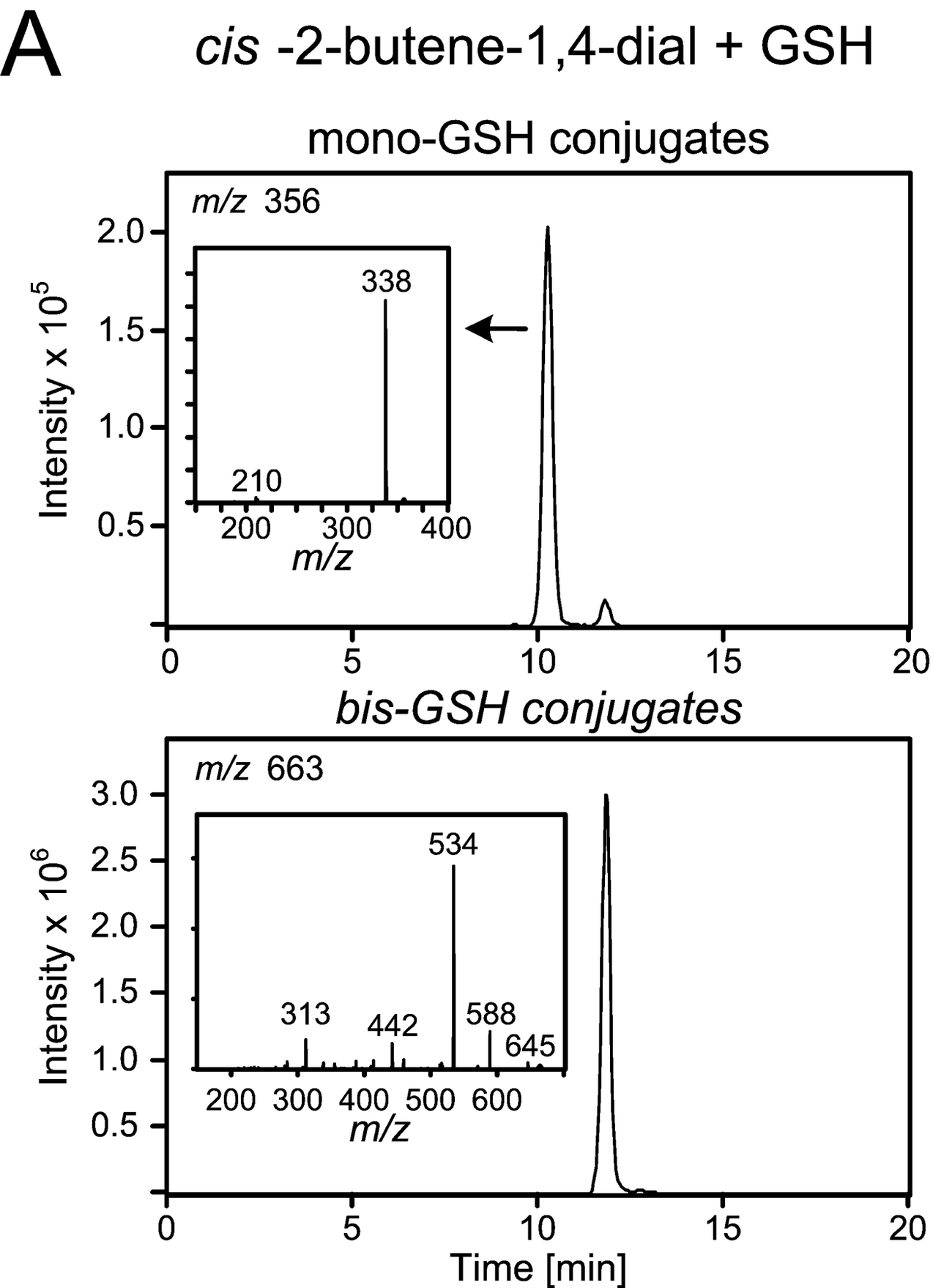


Figure 3



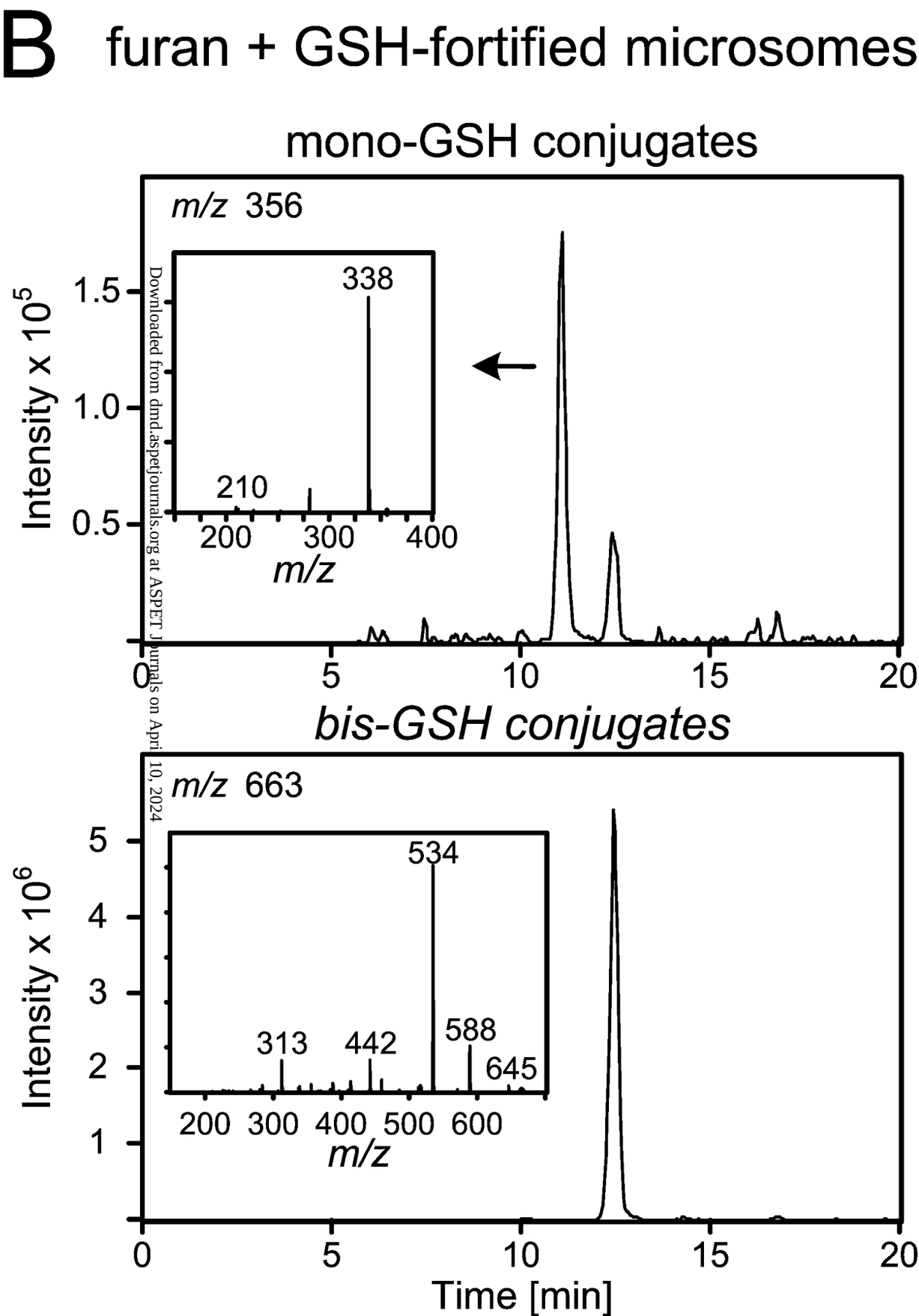


Figure 4

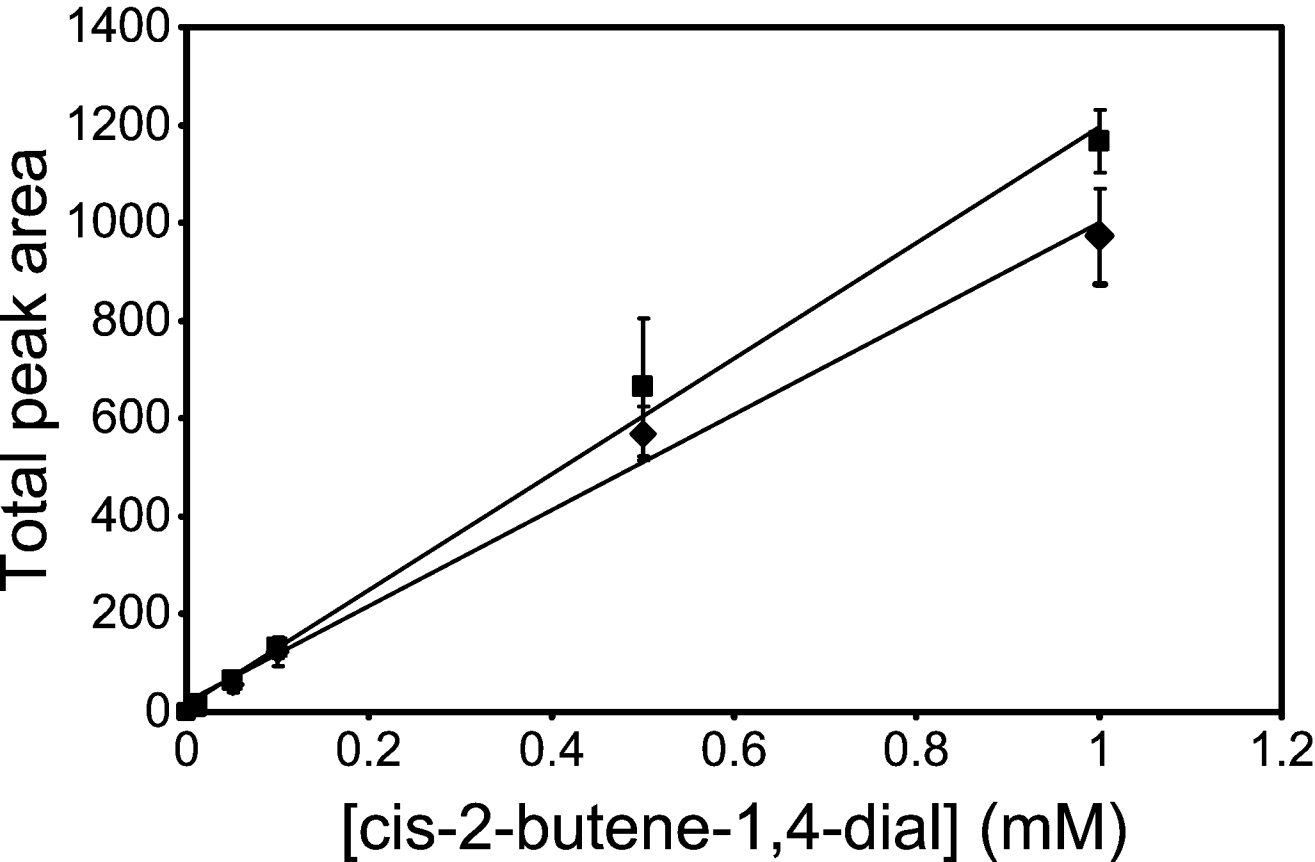
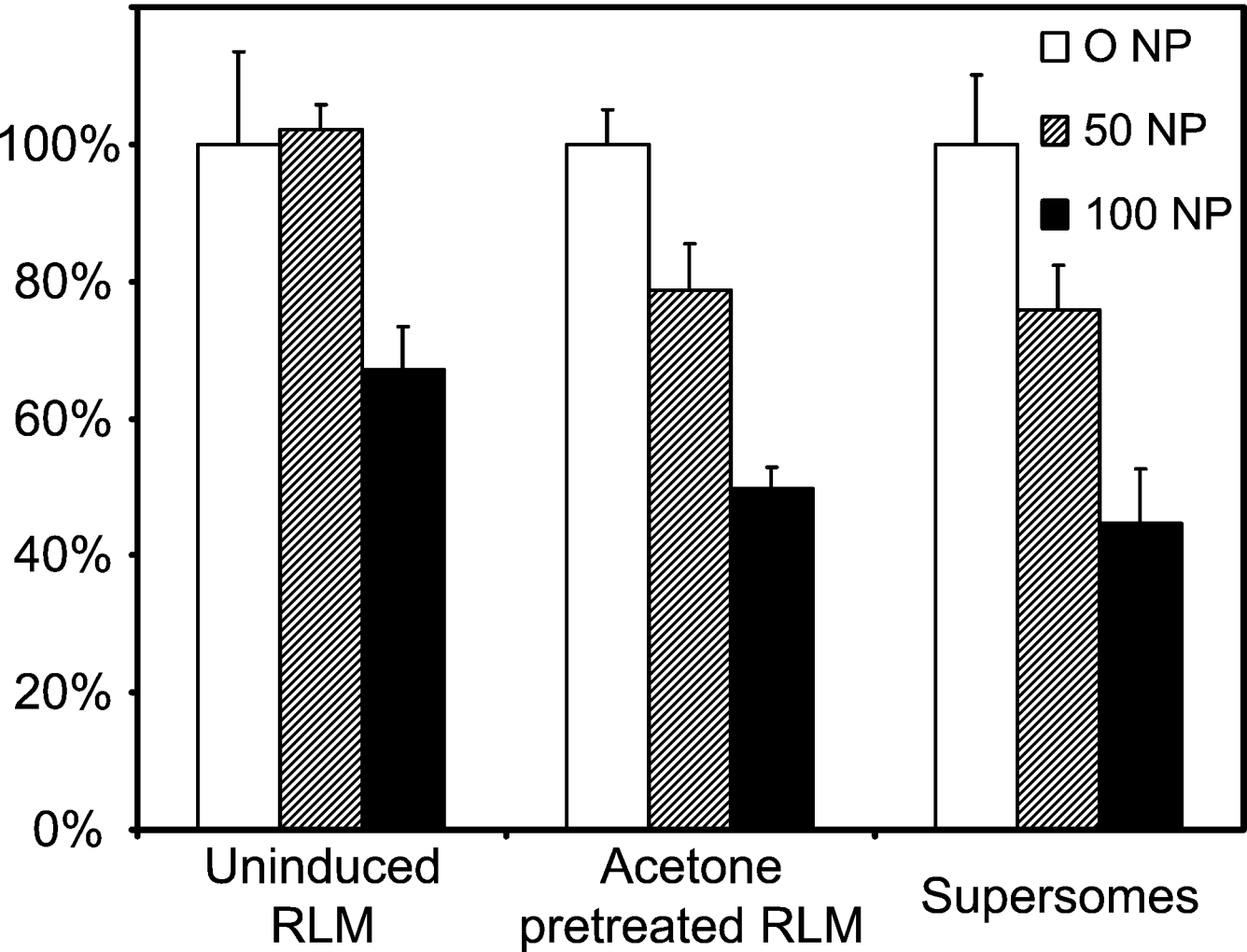

1400
1200
1000
800
600
400
200
0
Total peak area
0
0.2
0.4
0.6
0.8
1
1.2
[cis-2-butene-1,4-dial] (mM)

Figure 5


O NP
50 NP
100 NP
100%
80%
60%
40%
20%
0%
Uninduced RLM
Acetone pretreated RLM
Supersomes