

LACK OF SINGLE-DOSE DISULFIRAM EFFECTS ON CYTOCHROME P-450 2C9, 2C19, 2D6, AND 3A4 ACTIVITIES: EVIDENCE FOR SPECIFICITY TOWARD P-450 2E1

EVAN D. KHARASCH, DOUGLAS C. HANKINS, CAROLE JUBERT, KENNETH E. THUMMEL, AND JULIE K. TARADAY

Departments of Anesthesiology, Medicinal Chemistry, and Pharmaceutics, University of Washington, Seattle, Washington and the Anesthesiology Service, Puget Sound Veterans Affairs Health Care System, Seattle, Washington

(Received December 18, 1998; accepted February 23, 1999)

This paper is available online at <http://www.dmd.org>

ABSTRACT:

Disulfiram and its primary metabolite diethyldithiocarbamate are effective mechanism-based inhibitors of cytochrome P-450 2E1 (CYP2E1)¹ in vitro. Single-dose disulfiram diminishes CYP2E1 activity in vivo and has been used to identify CYP2E1 participation in human drug metabolism and prevent CYP2E1-mediated toxification. Specificity of single-dose disulfiram toward CYP2E1 in vivo, however, remains unknown. This investigation determined single-dose disulfiram effects on human CYP 2C9, 2C19, 2D6, and 3A4 activities in vivo. In four randomized crossover experiments, volunteers received isoform-selective probes (oral tolbutamide, mephenytoin, dextromethorphan, or i.v. midazolam) on two occasions, 10 h after oral disulfiram or after no pretreatment (controls). Plasma and/or urine parent and/or metabolite concentrations were measured by HPLC or gas chromatography-mass spectrometry. CYP2C9, 2C19, 2D6, and 3A4 activities were determined from the tolbutamide metabolic ratio, 4'-hydroxymephenytoin excretion,

and dextromethorphan/dextrorphan ratios in urine and midazolam systemic clearance, respectively. Midazolam clearance (670 ± 190 versus 700 ± 240 ml/min, disulfiram versus controls), dextromethorphan/dextrorphan metabolic ratio (0.013 ± 0.033 versus 0.015 ± 0.035), 4'-hydroxymephenytoin excretion (122 ± 22 versus 128 ± 25 μ mol), and tolbutamide metabolite excretion (577 ± 157 versus 610 ± 208 μ mol) were not significantly altered by disulfiram pretreatment, although the tolbutamide metabolic ratio was slightly diminished after disulfiram (60 ± 17 versus 81 ± 40 , $p < .05$). Results show that single-dose disulfiram does not cause clinically significant inhibition of human CYP2C9, 2C19, 2D6, and 3A4 activities in vivo. When single-dose disulfiram is used as an in vivo probe for P-450, inhibition of drug metabolism suggests selective involvement of CYP2E1. Single-dose disulfiram should not cause untoward drug interactions from inhibition of other P-450 isoforms.

The metabolism of numerous halogenated xenobiotics used in industrial applications, endogenous compounds, ethanol, and a few drugs such as chlorzoxazone, isoniazid, acetaminophen, and volatile anesthetics is catalyzed by human liver cytochrome P-450 (CYP) 2E1 (Lieber, 1997). Potential consequences of such metabolism include bioactivation resulting in toxification and/or carcinogenesis. One approach for identifying CYP2E1 participation in human xenobiotic biotransformation in vivo is to manipulate CYP2E1 activity, by induction or inhibition, and assess the effect on candidate drug metabolism. When bioactivation portends toxification, the latter approach is preferable. A corollary application of this technique is that CYP2E1 inhibition may be used prophylactically, or possibly postexposure, to prevent CYP2E1-mediated toxification.

Single-dose disulfiram inhibition of CYP2E1 has been explored as an in vivo probe for CYP2E1 participation in biotransformation and as a potential preventative agent against CYP2E1-mediated toxification. Disulfiram and its reduced metabolite diethyldithiocarbamate inhibit

human liver microsomal CYP2E1 in vitro (Guengerich et al., 1991) and rat CYP2E1 in vivo (Brady et al., 1991). Using chlorzoxazone 6-hydroxylation as a measure of CYP2E1 activity (Peter et al., 1990), disulfiram was found to reduce chlorzoxazone elimination clearance and 6-hydroxychlorzoxazone formation clearance to 15% and 7% of control values, respectively (Kharasch et al., 1993). This established single-dose disulfiram as an effective inhibitor of human CYP2E1 in vivo, a useful probe for delineating CYP2E1 participation in drug disposition and for potential prevention of CYP2E1-mediated toxification. Single-dose disulfiram was subsequently used to establish CYP2E1 participation in human in vivo metabolism of enflurane, sevoflurane, and halothane (Kharasch et al., 1994, 1995, 1996) and to diminish CYP2E1-mediated metabolism of halothane to potentially toxic reactive intermediates (Kharasch et al., 1996).

Although initial in vitro investigations indicated that disulfiram and diethyldithiocarbamate were selective, mechanism-based inhibitors of CYP2E1 (Guengerich et al., 1991), their selectivity was subsequently questioned. For example, diethyldithiocarbamate was also reported to inhibit human liver microsomal P-450s 2A6 (Yamazaki et al., 1992; Chang et al., 1994; Ono et al., 1996) and 2C19 (Ono et al., 1996) and, at higher concentrations, both 2C8 and 3A3/4 (Chang et al., 1994). Furthermore, although disulfiram efficacy toward CYP2E1 is unquestioned, the in vivo specificity of single-dose disulfiram for only CYP2E1 remains unknown. Accurate interpretation of clinical studies using disulfiram as a (presumably) selective inhibitor of CYP2E1 rests on this identification. Furthermore, before disulfiram can be

Supported by National Institutes of Health Grants R01 GM48712, P01 GM32165, and M01 RR00037 to the University of Washington Clinical Research Center.

¹ Abbreviation used is: TTCA, 2-thiothiazolidine-4-carboxylic acid.

Send reprint requests to: Evan D. Kharasch, Department of Anesthesiology, Box 356540, University of Washington, Seattle, WA 98195. E-mail: kharasch@u.washington.edu

recommended to prevent CYP2E1-mediated drug or xenobiotic bioactivation and toxicity, its safety, vis-a-vis the absence of unwanted and potentially hazardous non-CYP2E1 drug interactions, must be demonstrated. We recently showed that single-dose disulfiram does not inhibit human CYP2A6 activity (coumarin hydroxylation) in vivo (Kharasch et al., 1998). The present investigation determined the effect of single-dose disulfiram on the other human P-450 isoforms responsible for metabolizing the majority of therapeutically used drugs, CYPs 2C9, 2C19, 2D6, and 3A4 (Guengerich, 1995; Wrighton et al., 1996).

Materials and Methods

Patient Selection and Clinical Protocol. Male and nonpregnant female nonsmoking volunteers participated in this investigation after written informed consent was obtained. The investigational protocols were approved by the Institutional Human Subjects Committee. Not all subjects participated in each study pair; group size was determined by prospective power analysis. Subjects were in good health, within 20% of ideal body weight, had no history of hepatic or renal disease, and were taking no prescription medications (excluding a few subjects on oral contraceptives) during the investigation. Subjects abstained from caffeine, grapefruit, grapefruit juice, and ethanol beginning the day before each study day and continuing throughout the period of urine collection. Subjects also abstained from ethanol for 5 days after disulfiram administration. Each substrate probe was studied in a crossover design, with subjects randomly assigned first to the control or disulfiram phase and a washout period of 1 to 2 weeks between phases. Pretreated subjects received 500 mg of disulfiram orally at bedtime, 9 to 10 h before substrate probes, whereas controls received nothing.

CYP3A4 activity was assessed by the clearance of midazolam (Thummel et al., 1994a,b). Subjects received 1 mg i.v. midazolam, and venous blood samples were obtained through an indwelling catheter in the contralateral arm for 12 h after dosing. Plasma was frozen at -20°C for later analysis. Based on the population variability of midazolam disposition (Kassai et al., 1988), 15 subjects (30 ± 5 years, 72 ± 13 kg, 8:7 male/female) were studied to detect a 30% difference in midazolam clearance ($\alpha = 0.05$, $\beta = 80\%$). CYP3A4 activity was also estimated by the *N*-demethylation of dextromethorphan to 3-methoxymorphinan (Jacqz-Aigrain et al., 1993; Ducharme et al., 1996; Jones et al., 1996).

CYP2D6 activity was determined from the metabolism of dextromethorphan (Schmid et al., 1985). Subjects received 30 mg oral dextromethorphan hydrobromide ($85.5 \mu\text{mol}$ of dextromethorphan) with 200 ml of water after an overnight fast, followed 2 h later by a standard breakfast. Urine was collected 0 to 8 h after dextromethorphan, the volume was measured, and an aliquot was frozen at -20°C for metabolite analysis. Forty subjects (28 ± 5 years, 69 ± 12 kg, 20:20 male/female) were studied to detect a 30% difference in dextromethorphan *O*-demethylation ($\alpha = 0.05$, $\beta = 80\%$), based on the population variability of dextromethorphan *O*-demethylation and the expected incidence of poor metabolizers (Evans et al., 1993).

CYP2C19 activity was determined by the 4'-hydroxylation of *S*-mephenytoin (Wrighton et al., 1993), as described previously (Kupfer and Preisig, 1984; Wedlund et al., 1984). Subjects received racemic mephenytoin (100 mg orally with 200 ml of water; $229 \mu\text{mol}$ of *S*-mephenytoin), followed 2 h later by a standard breakfast. Urine was collected from 0 to 8 h after mephenytoin dosing, the volume was measured, and an aliquot was frozen at -20°C for metabolite analysis. Fifteen subjects (30 ± 5 years, 72 ± 13 kg, 8:7 male/female) were studied, based on the calculation that 13 subjects would be needed to detect a 30% difference in mephenytoin metabolism ($\alpha = 0.05$, $\beta = 80\%$) using the published population variability of mephenytoin hydroxylation and the expected incidence of poor metabolizers (Wilkinson et al., 1989; Goldstein et al., 1997).

CYP2C9 activity was assessed by the metabolism of tolbutamide to hydroxytolbutamide and its secondary metabolite carboxytolbutamide (Brian et al., 1989; Relling et al., 1990), as described previously (Peart et al., 1987; Veronese et al., 1990). Subjects received tolbutamide (500 mg orally, 1849 μmol) with 200 ml of water after breakfast. Urine was collected from 6 to 12 h after tolbutamide, the volume was measured, and an aliquot was frozen at -20°C for metabolite analysis. Subjects self-administered glucose tablets at

regular intervals until the midafternoon urine collection to prevent hypoglycemia. Twenty subjects (29 ± 4 years, 70 ± 12 kg, 9:11 male/female) were studied to detect a 30% difference in tolbutamide metabolic ratio ($\alpha = 0.05$, $\beta = 80\%$), based on the population variability of tolbutamide metabolism (Veronese et al., 1993).

Analytical Methods. Dextromethorphan, dextrorphan, 3-methoxymorphinan, 3-hydroxymorphinan, levallorphan, tolbutamide, hydroxytolbutamide, and 4'-hydroxymephenytoin were obtained from Research Biochemicals, Inc. (Natick, MA), carboxytolbutamide was purchased from UltraFine Chemicals (Manchester, England), and all other chemicals were from Sigma (St. Louis, MO).

Plasma midazolam concentrations were determined by gas chromatography-mass spectrometry as described previously (Thummel, 1994b), with minor modification. Briefly, 1.5 ml of plasma containing the internal standard diazepam (25 ng) and 0.5 ml of 1 M sodium hydroxide was extracted with 2×3 ml of ethyl acetate-heptane (1:1, v/v). The combined organic layers were evaporated under nitrogen at 40°C , reconstituted in $75 \mu\text{l}$ of acetate-heptane, and analyzed on a Hewlett-Packard 5890 series II GC/5972 mass selective detector using a DB-5 capillary column ($30 \text{ m} \times 0.32 \text{ mm} \times 0.25 \mu\text{m}$ film thickness) (J&W, Folsom, CA). The column head pressure (helium carrier gas) was 25 psi for 1 min and then decreased to 8 psi. The oven temperature was 200°C for 1 min, increased $10^{\circ}\text{C}/\text{min}$ to 280°C , and then to 320°C at $5^{\circ}\text{C}/\text{min}$. Injector and transfer line temperatures were 290°C and 300°C , respectively. Ions monitored were *m/z* 283.1 and 310.1 for diazepam and midazolam, respectively. Standard curves were prepared daily (0.1–25 ng/ml) and were linear ($r^2 > 0.99$ over 0.1–5 and 0.1–25 ng/ml). The interday variability was 12, 7, and 1% at 0.1, 5, and 25 ng/ml, respectively.

Urine dextromethorphan and metabolite concentrations were determined by HPLC with fluorescence detection as described (Chen et al., 1990), with the following modifications. Urine (1 ml) was incubated overnight at 37°C with 1 ml of 0.1 M potassium acetate buffer (pH 5) containing β -glucuronidase (5000 U/ml), spiked with $5 \mu\text{g}$ of levallorphan and diluted with 1 ml of saturated sodium carbonate. Samples were twice extracted with 3 ml of diethyl ether/chloroform/isopropanol (20:9:1) by vortexing for 10 min and centrifuged at $2000g$ for 10 min; organic layers were combined into a 15-ml tapered polypropylene screw cap tube containing $200 \mu\text{l}$ of 0.1 N HCl. Analytes were back-extracted by vortexing for 10 min and centrifuging ($2000g$, 10 min), and the organic layer was aspirated and the aqueous phase evaporated to dryness under nitrogen at 55°C . Samples were reconstituted in $100 \mu\text{l}$ of 0.1 N HCl, vortexed, and transferred to an autosampler vial. Chromatography was performed with a Hewlett Packard 1050 HPLC system coupled to a Kratos Spectroflow 980 fluorescence detector (excitation 228 nm, no emission cut-off filter) using a Rainin Microsorb phenyl column ($250 \times 4.6 \text{ mm}$, 5 microns). The mobile phase was methanol/acetonitrile/10 mM potassium phosphate (pH 3.5) (20:25:55) at 1.2 ml/min. Analyses were carried out at room temperature. Each sample was injected twice (1 and 10 μl) to ensure that peaks were within the linear range of the detector. Calibration standards were prepared daily using blank urine containing 80 to 10,000 ng/ml dextrorphan and 3-hydroxymorphinan and 8 to 1000 ng/ml dextromethorphan and 3-methoxymorphinan. Peak area ratios were used for analysis of dextromethorphan and dextrorphan, and 3-methoxymorphinan and 3-hydroxymorphinan were quantified from peak height ratios because complete baseline resolution was unattainable in some patients. Standard curves were linear over the concentration ranges used ($r^2 > 0.99$, 0.99, 0.98, and 0.96 for dextromethorphan, dextrorphan, 3-methoxymorphinan, and 3-hydroxymorphinan, respectively). Interday coefficients of variation were 25 and 2% at 16 and 1000 ng/ml dextromethorphan, 22 and 15% at 0.3 and 2.5 $\mu\text{g}/\text{ml}$ dextrorphan, 19 and 2% at 16 and 1000 ng/ml 3-methoxymorphinan, and 18 and 8% at 0.3 and 2.5 $\mu\text{g}/\text{ml}$ 3-hydroxymorphinan.

The concentration of 4'-hydroxymephenytoin in urine was measured by HPLC with UV detection as described, with minor modification (Xie et al., 1995). Urine (100 μl), water (900 μl), and 50 μl of 0.5 M acetate buffer (pH 5.0, containing 5000 U/ml β -glucuronidase) were incubated in polyethylene tubes at 37°C for 18 h. The hydrolysate was transferred to a glass tube containing $5 \mu\text{g}$ of phenobarbital and twice extracted by vortexing with 3 ml of diethyl ether. Following centrifugation ($2000g$, 10 min), the combined organic layers were evaporated to dryness at 45°C under nitrogen, reconstituted in 50 μl of mobile phase, and transferred to an autosampler vial. Chromatography was performed at room temperature on an HP 1050 HPLC

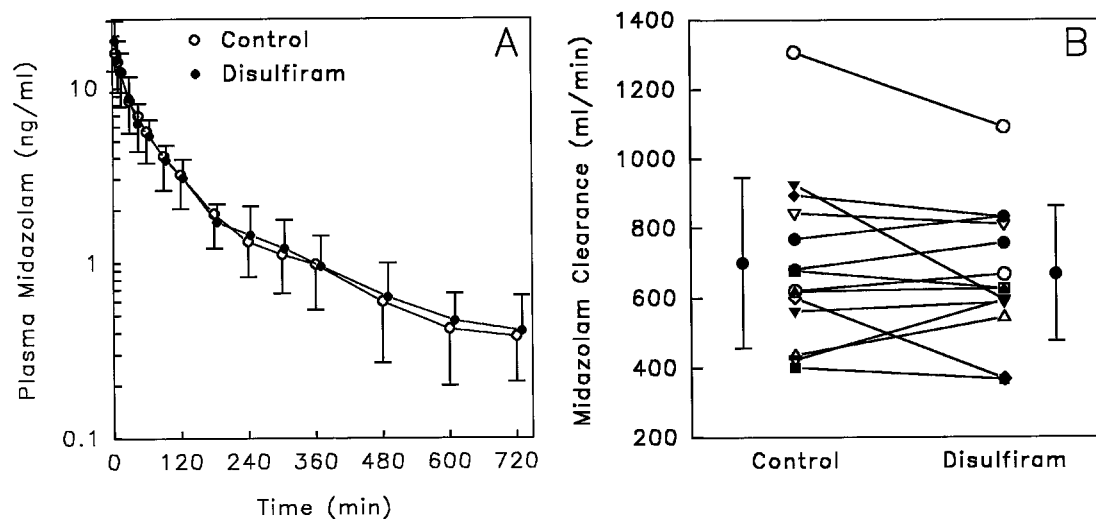


FIG. 1. Disulfiram effect on midazolam disposition. A, plasma midazolam concentrations (mean \pm S.D.) for all subjects. B, midazolam systemic clearance, shown for individual subjects and mean \pm S.D.

system with variable wavelength UV detector at 204 nm, using a Rainin Microsorb-MV C-18 analytical column (5 microns, 250 \times 4.6 mm) (Varian, Walnut Creek, CA) and Opti-Guard C-18 (1 mm) guard column (Optimize Technologies, Oregon City, OR). The mobile phase gradient began at 69:31 water/acetonitrile, increased linearly to 38:62 over 10 min, held for 2 min, returned to its initial composition over 3 min, and then re-equilibrated for 5 min. The flow rate was 1.75 ml/min. Injection volume was 20 μ l. Analyte stock solutions were prepared in acetonitrile and used to formulate 4'-hydroxymephenytoin standards in urine for calibration curves (0.5–25 μ g/ml, $r^2 = 0.999$). The intraday coefficient of variation for 4'-hydroxymephenytoin was 2% at 0.5 and 25 μ g/ml. All urine samples were analyzed on the same day.

Hydroxytolbutamide, carboxytolbutamide, and unchanged tolbutamide in urine were determined by HPLC after glucuronidase treatment using a modification of a previous method (Csillag et al., 1989). Briefly, urine (1 ml) containing 20 μ g of chlorpropamide (internal standard) and 100 μ l of 2 M HCl was twice extracted with 2 ml of diethyl ether by vortexing and was then centrifuged for 10 min at 2000g. The combined organic layers were evaporated to dryness under nitrogen at 40°C, reconstituted in 100 μ l of methanol/acetonitrile/isopropanol/0.1% phosphoric acid buffer (pH 2.0) (50:8.5:8.5:33) and transferred to autosampler vials. Analyses were performed at room temperature on an HP 1050 HPLC system with variable wavelength detector (235 nm) and a Rainin Microsorb-MV C-18 column (5 microns, 4.6 \times 250 mm) at room temperature. The isocratic mobile phase was acetonitrile/isopropanol/0.1% phosphoric acid (pH 2.0) (17:17:66) at 1.0 ml/min, and the injection volume was 10 μ l. Tolbutamide in urine was also analyzed by HPLC-electrospray mass spectrometry using an HP 1100 HPLC-MSD system and Rainin Microsorb C-18 analytical column (5 microns, 2.1 \times 150 mm) coupled to a HAIPEEK C-18 guard column. Injection volume was 5 μ l, and the isocratic mobile phase was acetonitrile/isopropanol/aqueous 0.5% trifluoroacetic acid (20:20:80) at 200 μ l/min. The mass spectrometer acquisition parameters included positive ionization, quadrupole temperature 99°C, gas temperature 350°C, 55 nA cap current, and drying gas at 10 liters/min. Tolbutamide and chlorpropamide were monitored at m/z 271.2 and 277.2 (MH⁺), respectively. Analyte stock solutions were prepared in methanol, and standard curves of peak area ratios were created daily using drug-free urine (5–115 μ g/ml hydroxytolbutamide and carboxytolbutamide, 0.5–5.0 μ g/ml tolbutamide) and used to quantify concentrations in patient samples. Standard curves were linear over the concentration ranges used ($r^2 > 0.995$ for all analytes), and the interday coefficient of variation was 8 and 10% for hydroxytolbutamide at 5 and 115 μ g/ml, 3 and 0.3% for carboxytolbutamide at 5 and 115 μ g/ml, and 7 and 2% for tolbutamide at 0.5 and 5 μ g/ml (intraday, liquid chromatography-mass spectrometry).

To verify that disulfiram had been ingested, subjects kept medication diaries, and urine was analyzed for 2-thiothiazolidine-4-carboxylic acid (TTCA), an established human biomarker for the major disulfiram metabolite

carbon disulfide and also for disulfiram itself (van Doorn et al., 1981, 1982). TTCA was synthesized, and TTCA in urine was analyzed by gas chromatography-mass spectrometry, as described previously (Johnson et al., 1996). Briefly, 1 ml of filtered urine and 0.8 ml of 1 N HCl were extracted with 2 ml of ethyl acetate, dried over magnesium sulfate, evaporated to dryness, dissolved in methanol, derivatized with diazomethane, evaporated to dryness, reconstituted in acetonitrile, and analyzed by selected ion mode gas chromatography-mass spectrometry (m/z 132; M⁺-COOCH₃). A linear standard curve was prepared using blank urine and TTCA (10–1000 ng/ml, $r^2 > 0.99$) and used for quantification. Urine samples were collected as described above and also for 0 to 24 h after midazolam.

Data Analysis. CYP2C9 activity was assessed by the tolbutamide metabolic ratio [(hydroxytolbutamide + carboxytolbutamide)/tolbutamide] in the 6 to 12-h urine (Veronese et al., 1990) and tolbutamide metabolite recovery (Peart, 1987). CYP2C19 activity was determined by the amount of 4'-hydroxymephenytoin excreted in the 0 to 8-h urine, expressed also as the hydroxylation index (μ mol 5-mephenytoin/ μ mol 4'-hydroxymephenytoin) (Kupfer and Preisig, 1984; Wedlund et al., 1984; Xie et al., 1997). Individuals excreting <2% of the dose as 4'-hydroxymephenytoin were considered phenotypically poor metabolizers (Wedlund et al., 1984). The molar dextromethorphan/dextrorphan metabolic ratio in 0 to 8-h urine was used to determine CYP2D6 activity (Schmid et al., 1985; Jacqz-Aigrain et al., 1993; Jones et al., 1996). A metabolic ratio ≥ 0.3 was considered evidence of CYP2D6 poor metabolizer phenotype (Schmid et al., 1985). Midazolam systemic clearance (dose/area under the curve) was determined by noncompartmental analysis (WinNonlin 1.5; Scientific Consulting, Inc. Cary, NC) and used as a measure of hepatic CYP3A4 activity (Thummel et al., 1994a, 1994b). Drug disposition in the control and disulfiram sessions was compared by paired *t* test or by Wilcoxon signed rank test if normality assumptions were violated. Significance was assigned at $p < .05$. Results are shown as the mean \pm standard deviation.

Results

Mean midazolam plasma concentration versus time profiles in control and disulfiram-treated subjects were superimposable (Fig. 1A). Systemic clearance (670 \pm 190 versus 700 \pm 240 ml/min, $p = .39$) was similarly unchanged by disulfiram (Fig. 1B).

Dextromethorphan disposition in untreated subjects resembled that in previous reports (Jacqz-Aigrain et al., 1993; Ducharme et al., 1996; Jones et al., 1996). Urine excretion (0–8 h) of dextromethorphan, 3-methoxymorphinan, 3-hydroxymorphinan, and dextromethorphan was 27 \pm 12, 0.05 \pm 0.06, 14 \pm 8, and 0.3 \pm 0.5 μ mol, respectively, representing 32 \pm 15, 0.06 \pm 0.07, 16 \pm 10, and 0.3 \pm 0.5% of the

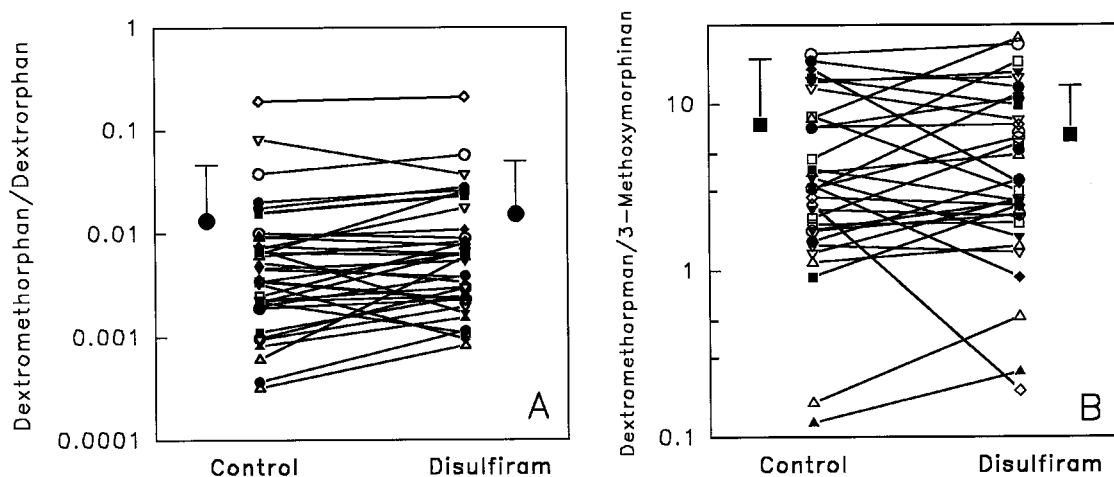


Fig. 2. Disulfiram effect on dextromethorphan metabolism. A, dextromethorphan/dextrorphan metabolic ratio (CYP2D6). B, dextromethorphan/3-methoxymorphinan metabolic ratio (CYP3A4).

dose, with $48 \pm 20\%$ metabolite recovery. Dextromethorphan *O*-demethylation was unaffected by disulfiram pretreatment (Fig. 2A). The mean dextromethorphan/dextrorphan metabolic ratio was unchanged by disulfiram (0.013 ± 0.033 versus 0.015 ± 0.035 , $p = .26$), and no subject was converted to a phenotypically poor metabolizer. There was an excellent correlation ($r = 0.94$, not shown) between the two ratio measures of CYP2D6 activity, dextromethorphan/dextrorphan and dextromethorphan/3-hydroxymorphinan, as described previously (Jones et al., 1996). The mean dextromethorphan/3-hydroxymorphinan metabolic ratio was also unchanged by disulfiram (0.028 ± 0.061 versus 0.032 ± 0.064 , $p = .22$, data not shown). Dextromethorphan *N*-demethylation, another putative marker of CYP3A4 activity, was also unaffected by disulfiram pretreatment, with no change observed in the dextromethorphan/3-methoxymorphinan metabolic ratio (Fig. 2B). Neither molar excretion of dextromethorphan and metabolites nor metabolite recovery was significantly different after disulfiram pretreatment.

Mephenytoin metabolism in controls was similar to that described previously (Kupfer and Preisig, 1984; Xie et al., 1997), averaging $128 \pm 25 \mu\text{mol}$ of 4'-hydroxymephenytoin ($56 \pm 11\%$ of the dose) excreted in 0 to 8-h urine, corresponding to a hydroxylation index of 1.9 ± 0.5 . One subject could not void in the control phase, and two subjects were found to be poor metabolizers; these were excluded from the statistical analysis. Single-dose disulfiram effects on mephenytoin metabolism are shown in Fig. 3. No subject was converted to a phenotypic poor metabolizer. Average 4'-hydroxymephenytoin excretion ($122 \pm 22 \mu\text{mol}$; hydroxylation index 1.9 ± 0.4) was unchanged compared with controls ($p = .72$). In one subject, 4'-hydroxymephenytoin control excretion appeared low, although s/he was not a poor metabolizer. Reanalysis without this subject showed that 4'-hydroxymephenytoin excretion was somewhat lower after disulfiram (120 ± 22 versus $134 \pm 18 \mu\text{mol}$; hydroxylation index 2.0 ± 0.4 versus 1.7 ± 0.2 , $p = .045$).

Tolbutamide metabolism in controls was similar to that described previously (Veronese et al., 1990, 1993), with one difference. Urine excretion (6–12 h) of hydroxytolbutamide ($104 \pm 37 \mu\text{mol}$; $6 \pm 2\%$ of the dose), carboxytolbutamide ($506 \pm 179 \mu\text{mol}$; $27 \pm 10\%$ of the dose), and percent of the dose recovered ($33 \pm 11\%$) were similar to those reported by Veronese et al. (4 ± 2 , 22 ± 8 , and $26 \pm 9\%$) (1993). In contrast, urinary tolbutamide excretion ($8.5 \pm 4.6 \mu\text{mol}$; $0.46 \pm 0.25\%$ of the dose) was higher than that reported previously ($0.49 \pm 0.23 \mu\text{mol}$; $0.027 \pm 0.013\%$ of the dose) (Veronese et al., 1993). Consequently, the 6 to 12-h tolbutamide metabolic ratio we

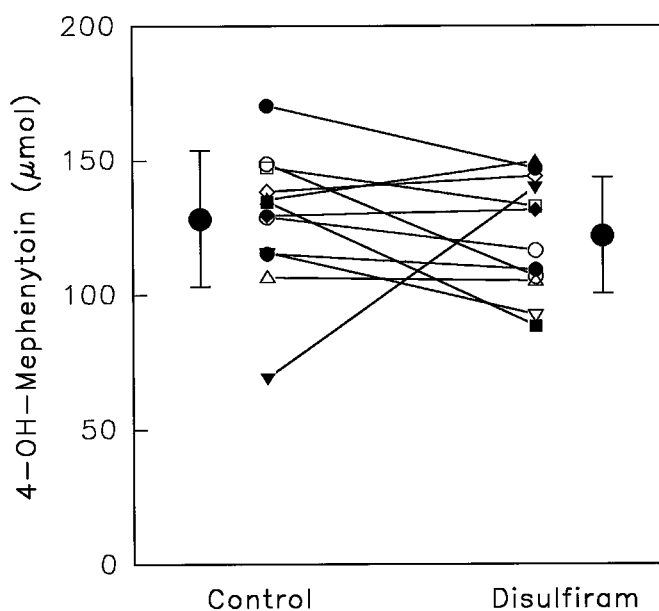


Fig. 3. Disulfiram effect on mephenytoin metabolism. Urine (0–8 h) 4'-hydroxymephenytoin excretion is shown.

observed (84 ± 39) was lower than those reported previously (794 ± 87 and 1144 ± 529) (Veronese et al., 1990, 1993). This difference may be related in part to assay conditions. Urine tolbutamide concentrations were close to the limit of quantification for the HPLC-UV assay, as reported previously (Veronese et al., 1990). Thus, urine tolbutamide was re-assayed using liquid chromatography-mass spectrometry, which afforded greater sensitivity and specificity. Concentrations measured by liquid chromatography-mass spectrometry were approximately 2-fold greater than when measured by HPLC-UV and were used to calculate the tolbutamide metabolic ratio reported.

The effect of single-dose disulfiram on tolbutamide metabolism is shown in Fig. 4. Tolbutamide metabolite excretion was unchanged (577 ± 157 versus $610 \pm 208 \mu\text{mol}$, $p = .54$) after disulfiram pretreatment (Fig. 4A). The tolbutamide metabolic ratio, however, was slightly, although significantly, diminished after disulfiram (60 ± 17 versus 81 ± 40 , $p = .032$), and this difference persisted after reanalysis without the subject with unusually low urine tolbutamide recovery (60 ± 18 versus 74 ± 26 , $p = .033$) (Fig. 4B).

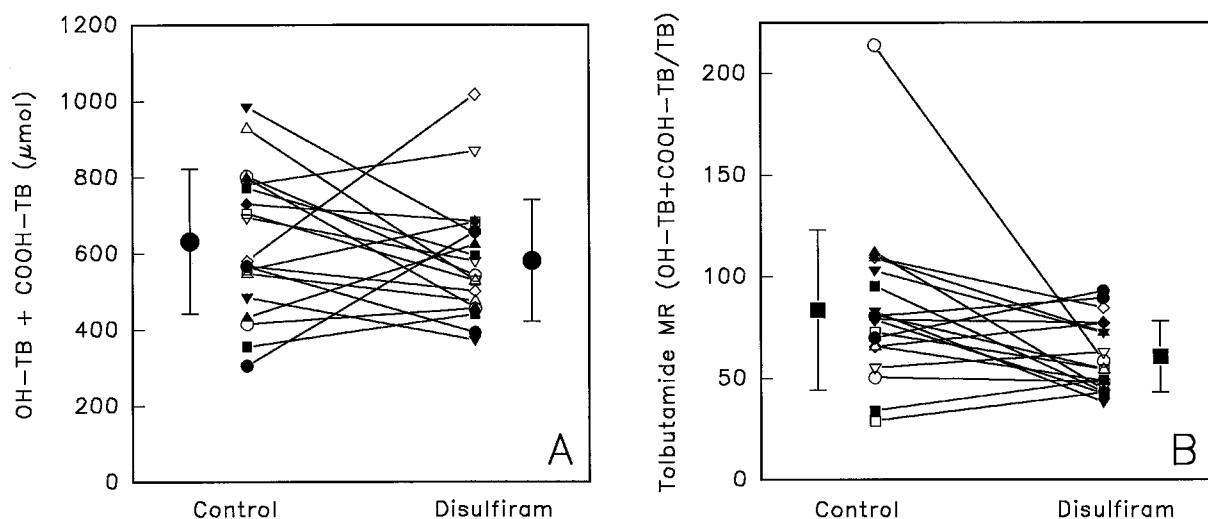


FIG. 4. Disulfiram effect on tolbutamide metabolism. A, urine (6–12 h) hydroxytolbutamide plus carboxytolbutamide molar excretion. B, urine (6–12 h) tolbutamide metabolic ratio. Disulfiram significantly lowered the metabolic ratio but not metabolite excretion.

TTCA excretion after control and disulfiram pretreatment is shown in Fig. 5 for each isoform probe. TTCA was present in urine of untreated subjects, as described previously, due presumably to dietary sources (Simon et al., 1994). All subjects instructed to ingest disulfiram the night before probe drug administration, with the exception of one subject in the dextromethorphan study, showed increased TTCA excretion.

Discussion

Single-dose disulfiram effects on CYP3A4 activity, assessed by systemic midazolam clearance and dextromethorphan *N*-demethylation, can be compared against the influence of known CYP3A4 inhibitors. Neither midazolam clearance nor the dextromethorphan/3-methoxymorphinan metabolic ratio was affected by disulfiram pretreatment. In contrast, known CYP3A4 inhibitors such as macrolide antibiotics and azole antifungals markedly impair *i.v.* midazolam clearance (Olkola et al., 1993, 1994; Kharasch et al., 1997). Furthermore, erythromycin pretreatment caused a significant 35 to 50% increase in the dextromethorphan/3-methoxymorphinan metabolic ratio (Ducharme et al., 1996; Jones et al., 1996). These results suggest that single-dose disulfiram does not significantly inhibit CYP3A4 activity *in vivo*. Lack of CYP3A4 inhibition, based on unaltered carbamazepine and methadone disposition, has also been seen with chronic disulfiram administration (Enghusen Poulsen et al., 1992).

Single-dose disulfiram effects on dextromethorphan *O*-demethylation can be compared against CYP2D6 activity in genetically deficient poor metabolizers and the effects of known CYP2D6 inhibitors. Phenotypic poor metabolizers have a dextrorphan/dextromethorphan metabolic ratio >0.3 (Schmid et al., 1985). In extensive metabolizers, the CYP2D6 inhibitor quinidine (1 week pretreatment) increased the dextrorphan/dextromethorphan metabolic ratio from 0.015 ± 0.061 to 1.9 ± 1.6 , and all subjects but one were converted from an extensive to a phenotypically poor metabolizer (Zhang et al., 1992). Similarly, a single quinidine dose administered to extensive metabolizers 12 h before dextromethorphan increased the average metabolic ratio from 0.005 to 0.4 (Schadel et al., 1995). In contrast, single-dose disulfiram did not significantly change the dextrorphan/dextromethorphan metabolic ratio, and no subject was converted from an extensive to a poor metabolizer. Thus, these data suggest that single-dose disulfiram does not significantly inhibit CYP2D6 activity *in vivo*.

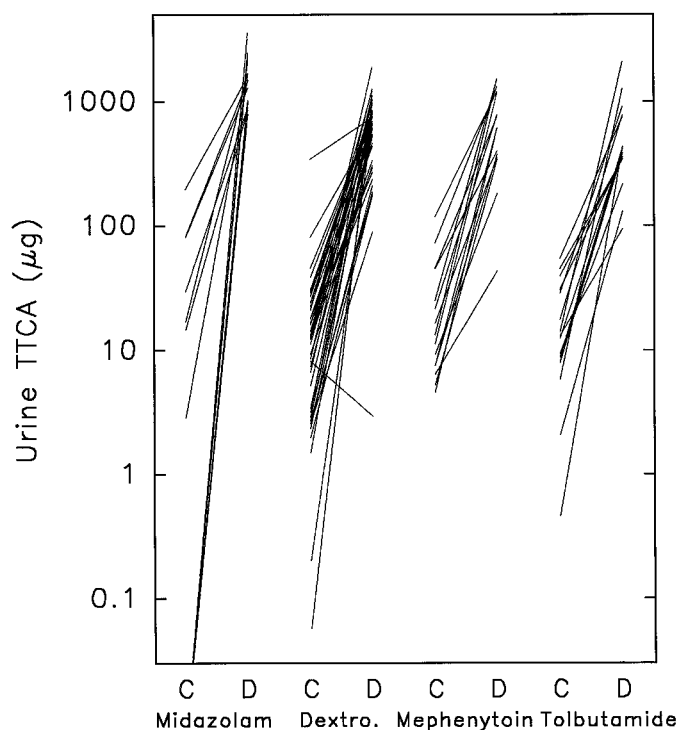


FIG. 5. Urine TTCA excretion in untreated control (C) and disulfiram (D) sessions for each isoform probe. Urine was collected for 0 to 24 h after midazolam, 0 to 8 h after dextromethorphan, 0 to 8 h after mephenytoin, and 6 to 12 h after tolbutamide, with disulfiram ingested approximately 10 h before the probe drugs.

Single-dose disulfiram effects on *S*-mephenytoin hydroxylation can be compared against CYP2C19 activity in genetically deficient poor metabolizers and the effects of known CYP2C19 alternate substrates. Poor metabolizers excrete less than 1 to 5% of *S*-mephenytoin as 4'-hydroxymephenytoin (<2–11 µmol) (Wedlund et al., 1984; Sohn et al., 1992; Kubota et al., 1996; Xie et al., 1997). A single dose of chloroguanide increased the mephenytoin hydroxylation index from 1.4 ± 0.2 to 2.5 ± 1.8 , corresponding to a reduction in 4'-hydroxymephenytoin excretion from 164 to 92 µmol (Partovian et al., 1995). A single dose of mephobarbital, administered shortly before mephenytoin, decreased *S*-mephenytoin clearance by approximately one-third

(Jacqz et al., 1986). Chronic omeprazole treatment decreased urine 4'-hydroxymephenytoin excretion from 123 ± 16 to $74 \pm 19 \mu\text{mol}$ (Caraco et al., 1996). In contrast to these 35 to 45% decreases in apparent CYP2C19 activity, single-dose disulfiram did not significantly change urine 4'-hydroxymephenytoin excretion, and, if the subject with low control 4'-hydroxymephenytoin excretion was omitted, it caused only an 11% decrease. Thus, these data suggest that single-dose disulfiram does not cause clinically significant inhibition of CYP2C19 activity in vivo.

Single-dose disulfiram effects on tolbutamide metabolism can be compared against the effects of known CYP2C9 inhibitors. Sulfaphenazole decreased plasma tolbutamide clearance by 80%, caused a 2-fold decrease in urine hydroxytolbutamide and carboxytolbutamide excretion, a 3-fold increase in tolbutamide excretion, and reduced the 6 to 12-h tolbutamide metabolic ratio to one-sixth that of controls (Veronese et al., 1990). In contrast, disulfiram had no significant effect on urine tolbutamide or metabolite excretion and caused only a 20% overall decrease in the metabolic ratio. These results suggest that single-dose disulfiram does not cause clinically significant inhibition of CYP2C9 activity in vivo. Chronic disulfiram administration also had no effect on tolbutamide clearance (Svendsen et al., 1976).

The observed lack of disulfiram effect on CYP 2C9, 2C19, 2D6, and 3A4 activities cannot be attributed to a failure of subjects to ingest the drug as instructed. Both the medication diaries and the urine monitoring clearly demonstrated disulfiram ingestion. Thus, single-dose disulfiram was administered but had minimal or no inhibitory effect on the above isoforms.

Effects of single-dose disulfiram differ from those of chronic disulfiram administration. Single-dose disulfiram diminishes CYP2E1 activity by more than 90% (Kharasch et al., 1993), and inhibition is relatively selective for this isoform. Daily (>4 days) disulfiram use did not alter CYP2C9 or CYP3A4 activities but did decrease the clearances of antipyrine, theophylline, caffeine, phenytoin, diazepam, and chlorthalidone (Enghusen Poulsen et al., 1992), suggesting inhibition of P-450s 1A2 and 2C19. However, even daily use decreased CYP1A2 (caffeine and theophylline clearance) and 2C19 (phenytoin clearance) activities by only 30 to 35% (Svendsen et al., 1976; Beach et al., 1986; Loi et al., 1989). Differences between single-dose and chronic disulfiram effects on extent and selectivity of inhibition likely result from the relative selectivity of disulfiram and diethyldithiocarbamate toward CYP2E1 (Guengerich et al., 1991), combined with rapid disulfiram elimination, which reduces inhibitor concentrations at the time of study (10 h after disulfiram dosing) (Petersen, 1992), thereby maximizing mechanism-based and minimizing competitive components of P-450 inhibition. Consistent with this hypothesis is the observation that antipyrine clearance was diminished 10, 16, and 32% after 1, 3, and 5 days of disulfiram (Loft et al., 1986). Thus, single-dose disulfiram has unique, isoform-selective inhibitory characteristics. These conclusions pertain only to mechanism-based inhibition, as competitive components of disulfiram interactions were not evaluated.

Results of the current investigation, together with previous observations that single-dose disulfiram did not significantly diminish CYP2A6 activity (coumarin hydroxylation) (Kharasch et al., 1998), while profoundly inhibiting chlorzoxazone 6-hydroxylation (Kharasch et al., 1993), demonstrate that single-dose disulfiram is a highly effective and selective inhibitor of human CYP2E1 activity in vivo. These results also support the selectivity of chlorzoxazone as an in vivo human CYP2E1 probe. There are two implications of the single-dose disulfiram attribute of CYP2E1 selectivity with respect to clinical P-450 isoform typing and therapeutic interventions. First, single-dose disulfiram inhibition of candidate drug disposition suggests

involvement of CYP2E1 but not CYPs 2A6, 2C9, 2C19, 2D6, and 3A4. Second, single-dose disulfiram use to prevent CYP2E1-mediated drug or xenobiotic bioactivation is unlikely to result in untoward drug interactions resulting from inhibition of other P-450 isoforms and potentially hazardous alteration in therapeutic drug disposition.

Acknowledgment. We thank Tauri Senn for determination of midazolam pharmacokinetic parameters.

References

- Beach CA, Mays DC, Guiler RC, Jacober CH and Gerber N (1986) Inhibition of elimination of caffeine by disulfiram in normal subjects and recovering alcoholics. *Clin Pharmacol Ther* **39**:265–270.
- Brady JF, Xiao F, Wang M-H, Li Y, Ning SM, Gapac JM and Yang CS (1991) Effects of disulfiram on hepatic P450IIE1, other microsomal enzymes, and hepatotoxicity in rats. *Toxicol Appl Pharmacol* **108**:366–373.
- Brian WR, Srivastava PK, Umbenhauer DR, Lloyd RS and Guengerich FP (1989) Expression of a human liver cytochrome P-450 protein with tolbutamide hydroxylase activity in *Saccharomyces cerevisiae*. *Biochemistry* **28**:4993–4999.
- Caraco Y, Wilkinson GR and Wood AJ (1996) Differences between white subjects and Chinese subjects in the in vivo inhibition of cytochrome P450s 2C19, 2D6, and 3A by omeprazole. *Clin Pharmacol Ther* **60**:396–404.
- Chang TKH, Gonzalez FJ and Waxman DJ (1994) Evaluation of triacetyloleandomycin, α -naphthoflavone and diethyldithiocarbamate as selective chemical probes for inhibition of human cytochromes P450. *Arch Biochem Biophys* **311**:437–442.
- Chen ZR, Somogyi AA and Bochner F (1990) Simultaneous determination of dextromethorphan and three metabolites in plasma and urine using high-performance liquid chromatography with application to their disposition in man. *Ther Drug Monit* **12**:97–104.
- Csillag K, Vereczkey L and Gachalyi B (1989) Simple high-performance liquid chromatographic method for the determination of tolbutamide and its metabolites in human plasma and urine using photodiode-array detection. *J Chromatogr* **490**:355–363.
- Ducharme J, Abdullah S and Wainer IW (1996) Dextromethorphan as an in vivo probe for the simultaneous determination of CYP2D6 and CYP3A activity. *J Chromatogr B* **678**:113–128.
- Enghusen Poulsen H, Loft S, Andersen JR and Andersen M (1992) Disulfiram therapy: Adverse drug reactions and interactions. *Acta Psychiatr Scand* **86** (Suppl 369):59–66.
- Evans WE, Relling MV, Rahman A, McLeod HL, Scott EP and Lin J-S (1993) Genetic basis for a lower prevalence of deficient CYP2D6 oxidative drug metabolism phenotypes in black Americans. *J Clin Invest* **91**:2150–2154.
- Goldstein JA, Ishizaki T, Chiba K, de Moraes SMF, Bell D, Krahn PM and Price Evans DA (1997) Frequencies of the defective CYP2C19 alleles responsible for the mephenytoin poor metabolizer phenotype in various Oriental, Caucasian, Saudi Arabian and American black populations. *Pharmacogenetics* **7**:59–64.
- Guengerich FP (1995) Human cytochrome P450 enzymes, in *Cytochrome P450: Structure, Mechanism and Biochemistry* (Ortiz de Montellano PR ed), pp 473–535. Plenum Press, New York.
- Guengerich FP, Kim D-H and Iwasaki M (1991) Role of human P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol* **4**:168–179.
- Jacqz-Aigrain E, Funck-Brentano C and Cresteil T (1993) CYP2D6-dependent and CYP3A-dependent metabolism of dextromethorphan in humans. *Pharmacogenetics* **3**:197–204.
- Jacqz E, Hall SD, Branch RA and Wilkinson GR (1986) Polymorphic metabolism of mephenytoin in man: Pharmacokinetic interaction with a co-regulated substrate, mephobarbital. *Clin Pharmacol Ther* **39**:646–653.
- Johnson DJ, Graham DG, Amarnath V, Amarnath K and Vanentine WM (1996) The measurement of 2-thiothiazolidine-4-carboxylic acid as an index of the in vivo release of CS₂ by dithiocarbamates. *Chem Res Toxicol* **9**:910–916.
- Jones DR, Gorski JC, Haehner BD, O'Mara EM and Hall SD (1996) Determination of cytochrome P450 3A4/5 activity in vivo with dextromethorphan N-demethylation. *Clin Pharmacol Ther* **60**:374–384.
- Kassai A, Toth G, Eichelbaum M and Klotz U (1988) No evidence of a genetic polymorphism in the oxidative metabolism of midazolam. *Clin Pharmacokinetics* **15**:319–325.
- Kharasch ED, Armstrong AS, Gunn K, Artru A and Cox K (1995) Clinical sevoflurane metabolism and disposition: II. The role of cytochrome P450 2E1 in fluoride and hexafluoroisopropanol formation. *Anesthesiology* **82**:1379–1388.
- Kharasch ED, Hankins DC, Baxter PJ and Thummel KE (1998) Single-dose disulfiram does not inhibit CYP2A6 activity. *Clin Pharmacol Ther* **64**:39–45.
- Kharasch ED, Hankins D, Mautz D and Thummel KE (1996) Identification of the enzyme responsible for oxidative halothane metabolism: Implications for prevention of halothane hepatitis. *Lancet* **347**:1367–1371.
- Kharasch ED, Russell M, Mautz D, Thummel KE, Kunze KL, Bowdle TA and Cox K (1997) The role of cytochrome P450 3A4 in alfentanil clearance: Implications for interindividual variability in disposition and perioperative drug interactions. *Anesthesiology* **87**:36–50.
- Kharasch ED, Thummel KE, Mautz D and Bosse S (1994) Clinical enflurane metabolism by cytochrome P450 2E1. *Clin Pharmacol Ther* **55**:434–440.
- Kharasch ED, Thummel K, Mhyre J and Lillibridge J (1993) Single-dose disulfiram inhibition of chlorzoxazone metabolism: A clinical probe for P450 2E1. *Clin Pharmacol Ther* **53**:643–650.
- Kubota T, Chiba K and Ishizaki T (1996) Genotyping of S-mephenytoin 4'-hydroxylation in an extended Japanese population. *Clin Pharmacol Ther* **60**:661–666.
- Kupfer A and Preisig R (1984) Pharmacogenetics of mephenytoin: A new drug hydroxylation polymorphism in man. *Eur J Clin Pharmacol* **26**:753–759.
- Lieber CS (1997) Cytochrome P-4502E1: Its physiological and pathological role. *Physiol Rev* **77**:517–544.
- Loft S, Sonne J, Pilsgaard H, Dossing M and Poulsen E (1986) Inhibition of antipyrine elimination by disulfiram and cimetidine: The effect of concomitant administration. *Br J Clin Pharmacol* **21**:75–77.
- Loi C-M, Day JD, Jue SG, Bush ED, Costello P, Dewey LV and Vestal RE (1989) Dose-

- dependent inhibition of theophylline metabolism by disulfiram in recovering alcoholics. *Clin Pharmacol Ther* **45**:476–486.
- Olkola KT, Aranko K, Luurila H, Hiller A, Saarnivaara L, Himberg JJ and Neuvonen PJ (1993) A potentially hazardous interaction between erythromycin and midazolam. *Clin Pharmacol Ther* **53**:298–305.
- Olkola KT, Backman JT and Neuvonen PJ (1994) Midazolam should be avoided in patients receiving the systemic antimycotics ketoconazole or itraconazole. *Clin Pharmacol Ther* **55**:481–485.
- Ono S, Hatanaka T, Hotta H, Satoh T, Gonzalez FJ and Tsutsui M (1996) Specificity of substrate and inhibitor probes for cytochrome P450s: Evaluation of *in vitro* metabolism using cDNA-expressed human P450s and human liver microsomes. *Xenobiotica* **26**:681–693.
- Partovian C, Jacqz-Aigrain E, Keundjian A, Jaillon P and Funck-Brentano C (1995) Comparison of chloroguanide and mephenytoin for the *in vivo* assessment of genetically determined CYP2C19 activity in humans. *Clin Pharmacol Ther* **58**:257–263.
- Pearl GF, Boutagy J and Shenfield GM (1987) Lack of relationship between tolbutamide metabolism and debrisoquine oxidation phenotype. *Eur J Clin Pharmacol* **33**:397–402.
- Peter R, Bocker R, Beaune PH, Iwasaki M, Guengerich FP and Yang CS (1990) Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. *Chem Res Toxicol* **3**:565–573.
- Petersen EN (1992) The pharmacology and toxicology of disulfiram and its metabolites. *Acta Psychiatr Scand* **86** (Suppl. 369):7–13.
- Relling MV, Aoyama T, Gonzalez FJ and Meyer UA (1990) Tolbutamide and mephenytoin hydroxylation by human cytochrome-P450s in the CYP2C subfamily. *J Pharmacol Exp Ther* **252**:442–447.
- Schadel M, Wu D, Otton SV, Kalow W and Sellers EM (1995) Pharmacokinetics of dextromethorphan and metabolites in humans: Influence of the CYP2D6 phenotype and quinidine inhibition. *J Clin Psychopharmacol* **15**:263–269.
- Schmid B, Bircher J, Preisig R and Kupfer A (1985) Polymorphic dextromethorphan metabolism: Co-segregation of oxidative O-demethylation with debrisoquin hydroxylation. *Clin Pharmacol Ther* **38**:618–624.
- Simon P, Nicot T and Dieudonné M (1994) Dietary habits, a non-negligible source of 2-thiothiazolidine-4-carboxylic acid and possible overestimation of carbon disulfide exposure. *Int Arch Occup Environ Health* **66**:85–90.
- Sohn DR, Kusaka M, Ishizaki T, Shin SG, Jang IJ, Shin JG and Chiba K (1992) Incidence of S-mephenytoin hydroxylation deficiency in a Korean population and the interphenotypic differences in diazepam pharmacokinetics. *Clin Pharmacol Ther* **52**:160–169.
- Svensden TL, Kristensen MB, Hansen JM and Skovsted L (1976) The influence of disulfiram on the half-life and metabolic clearance rate of diphenylhydantoin and tolbutamide in man. *Eur J Clin Pharmacol* **9**:439–441.
- Thummel KE, Shen DD, Podoll T, Kunze KL, Trager WF, Bacchi CE, Marsh CL, McVicar JP, Barr DM, Perkins JD and Carithers RL (1994a) Use of midazolam as a human cytochrome P450 3A probe: II. Characterization of inter- and intra-individual hepatic CYP3A variability following liver transplantation. *J Pharmacol Exp Ther* **271**:557–566.
- Thummel KE, Shen DD, Podoll T, Kunze KL, Trager WF, Hartwell P, Raisys V, Marsh CL, McVicar JP, Barr DM, Perkins JD and Carithers RL (1994b) Use of midazolam as a human cytochrome P450 3A probe: I. *In vitro-in vivo* correlations in liver transplant patients. *J Pharmacol Exp Ther* **271**:549–556.
- van Doorn R, Delbressine LPC, Leijdekkers C-M, Vertin PG and Henderson PT (1981) Identification and determination of 2-thiothiazolidine-4-carboxylic acid in urine of workers exposed to carbon disulfide. *Arch Toxicol* **47**:51–58.
- van Doorn R, Leijdekkers C-M, Nossent SM and Henderson PT (1982) Excretion of TTCA in human urine after administration of disulfiram. *Toxicol Lett* **12**:59–64.
- Veronese ME, Miners JO, Randles D, Gregov D and Birkett DJ (1990) Validation of the tolbutamide metabolic ratio for population screening with use of sulfaphenazole to produce model phenotypic poor metabolizers. *Clin Pharmacol Ther* **47**:403–411.
- Veronese ME, Miners JO, Rees DLP and Birkett DJ (1993) Tolbutamide hydroxylation in humans: Lack of bimodality in 106 healthy subjects. *Pharmacogenetics* **3**:86–93.
- Wedlund PJ, Aslanian WS, McAllister CB, Wilkinson GR and Branch RA (1984) Mephenytoin hydroxylation deficiency in Caucasians: Frequency of a new oxidative drug metabolism polymorphism. *Clin Pharmacol Ther* **36**:773–780.
- Wilkinson GR, Guengerich FP and Branch RA (1989) Genetic polymorphism of S-mephenytoin hydroxylation. *Pharmacol Ther* **43**:53–76.
- Wrighton SA, Stevens JC, Becker GW and Vandenbranden M (1993) Isolation and characterization of human liver cytochrome P450 2C19: Correlation between 2C19 and S-mephenytoin-4'-hydroxylation. *Arch Biochem Biophys* **306**:240–245.
- Wrighton SA, VandenBranden M and Ring BJ (1996) The human drug metabolizing cytochromes P450. *J Pharmacokinetic Biopharm* **24**:461–473.
- Xie H-G, Huang S-L, Xu Z-H, Xiao Z-S, He N and Zhou H-H (1997) Evidence for the effect of gender on activity of (S)-mephenytoin 4'-hydroxylase (CYP2C19) in a Chinese population. *Pharmacogenetics* **7**:115–119.
- Xie H-G, Huang S-L and Zhou H-H (1995) High-performance liquid chromatographic determination of urinary 4'-hydroxymephenytoin, a metabolic marker for the hepatic enzyme CYP2C19, in humans. *J Chromatogr B* **668**:125–131.
- Yamazaki H, Inui Y, Yun C-H, Guengerich FP and Shimada T (1992) Cytochrome P450 2E1 and 2A6 enzymes as major catalysts for metabolic activation of N-nitrosodialkylamines and tobacco-related nitrosamines in human liver microsomes. *Carcinogenesis* **13**:1789–1794.
- Zhang Y, Britto MR, Valderhaug KL, Wedlund PJ and Smith RA (1992) Dextromethorphan: Enhancing its systemic availability by way of low-dose quinidine-mediated inhibition of cytochrome P4502D6. *Clin Pharmacol Ther* **51**:647–655.