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Chlorzoxazone metabolism is increased in fasted Sprague-Dawley rats

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Abstract

Earlier data showed that men fasted for 38 h had a reduced rate of chlorzoxazone metabolism, suggesting a decreased level of cytochrome P450 2E1 (CYP2E1). In contrast, the level of CYP2E1 in fasted rats had been shown to be elevated. In this study, we have investigated whether chlorzoxazone metabolism in fasted rats was changed by determining the pharmacokinetics of chlorzoxazone and its metabolite, 6-hydroxychlorzoxazone (6-OHCZ), as a CYP2E1 probe, and by measuring liver CYP2E1 using immunoblot techniques. Chlorzoxazone was administered by gavage (50 mg kg^{-1}) or intravenously (25 mg kg^{-1}) to control (nine for oral and three for intravenous) and 24 h-fasted (nine for oral and four for intravenous) male Sprague-Dawley rats. Following sampling of blood through a jugular vein cannula, chlorzoxazone and 6-OHCZ plasma concentrations were measured by HPLC with UV detection. Pharmacokinetic parameters for chlorzoxazone and 6-OHCZ in each treatment group were determined by model fitting and non-compartmental analysis. In parallel with the increased liver CYP2E1 level, the elimination of chlorzoxazone and 6-OHCZ was significantly increased in fasted rats in the oral and the intravenous study. A multiple analysis of variance covariance analysis and a multiple regression analysis revealed a significant correlation between 1/t½ and CYP2E1 level and aniline hydroxylase activity. However, the correlation between 1/t½ and pentoxyresorufin *O*-dealkylase, ethoxyresorufin *O*-dealkylase and erythromycin *N*-demethylase was not significant. Therefore the contribution of other P450s to chlorzoxazone metabolism seemed to be minor in the concentration range that we tested. In conclusion, fasting rats for 24 h caused a measurable induction of CYP2E1, which produced a significant increase in the rate of chlorzoxazone metabolism and elimination.

Introduction

Cytochrome P450 2E1 (CYP2E1) is one of the multigene family enzymes involved in the metabolism of numerous exogenous and endogenous compounds, such as ethanol, long-chain fatty acids, certain nitrosamines, and therapeutic agents such as chlorzoxazone and paracetamol (Koop & Coon 1986; Raucy et al 1989; Peter et al 1990). It is also responsible for the toxicity of many compounds by creating highly reactive metabolites (Yang et al 1990; Guengerich et al 1991). Regulation of CYP2E1 has been shown to occur at various levels including gene transcription, mRNA stabilization, and protein stabilization (Song et al 1986; Roberts et al 1995a). Fasting for 24 to 48 h has been shown to induce CYP2E1 mRNA and protein concentrations 1.5- to 3-fold in the Sprague-Dawley rat (Hong et al 1987).

Since publication of the original report that hydroxylation of chlorzoxazone was catalyzed specifically by human liver CYP2E1 (Peter et al 1990), the rate of metabolism of chlorzoxazone has been increasingly used as a marker for CYP2E1 liver concentration (Chen & Yang 1996; Lucas et al 1999). In most cases, chlorzoxazone metabolism has changed in the direction that was expected based on the experimental treatment (e.g. increased during alcohol withdrawal (Dupont et al 1998), decreased following CYP2E1 suicide substrate (Emery et al 1999)). However, in a study of men fasted for 36 h, the elimination of chlorzoxazone was decreased, not increased as expected (O'Shea et al 1994). Since the human CYP2E1 levels were not determined in the study, the explanation for the decreased chlorzoxazone metabolism remains unclear. Therefore, we decided to investigate chlorzoxazone metabolism in fasted rats to see how chlorzoxazone metabolism

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changed. We investigated whether changes in the CYP2E1 liver concentration was parallel to the changes in chlorzoxazone metabolism in fasted rats. Although some studies demonstrated that the metabolism of chlorzoxazone was determined by CYP2E1 level (Peter et al 1990; Lucas et al 1999), others suggested that other P450s such as CYP3A4 and CYP1A could play a role in chlorzoxazone 6-hydroxylation in in-vitro studies (Ono et al 1995; Gorski et al 1997; Shimada et al 1999) and doubted the use of chlorzoxazone as a specific probe for CYP2E1. Therefore, we determined the correlation of in-vivo chlorzoxazone elimination with some other P450 enzyme activity to determine which P450s might be related to chlorzoxazone metabolism.

Materials and Methods

Materials

Chlorzoxazone, pentoxifylline, β-glucuronidase (from *Helix pomatia*), and horseradish peroxidase conjugated anti-goat IgG were purchased from Sigma Chemical Co. (St Louis, MO, USA). 6-Hydroxychlorzoxazone (6-OHCZ) was purchased from RBI (Natick, MA, USA). Acetonitrile was of HPLC grade and obtained from Fisher Scientific (Fair Lawn, NJ, USA). Goat anti-rat CYP2E1 polyclonal antibody was purchased from Gentest (Woburn, MA, USA). All other chemicals and solvents were of the highest grade of commercially available materials.

Animals and treatment

Male, Sprague-Dawley rats (340–420 g) with jugular cannula were obtained from Taconic Farms (Germantown, NY, USA) and maintained according to NIH Animal Care and Use Guidelines. All procedures were approved by the NIAAA Animal Care and Use Committee. The jugular cannula was secured using a head cap. Rat chow and tap water were freely available. All rats were maintained on a standard 12-h light/dark cycle. The animals were acclimated for a minimum of three days in a cage with bedding before being randomly assigned to either the control or fasted group. At approximately 0830 h food was removed from the control group, 4 h before chlorzoxazone administration. For the fasted group, food was removed at approximately 0830 h the day before chlorzoxazone administration.

Chlorzoxazone (10 mg mL^{-1}) was dissolved in saline with the aid of 0.1 M NaOH. Oral doses were given by gavage. Intravenous doses were given through the jugular cannula; after dosing the tubing attached to the head cap was removed and replaced with fresh tubing for sampling. Chlorzoxazone doses were administered at approximately 1300 h, approximately 15 min after the rats were given 8 U heparin (i.v.). For oral administration, blood samples (0.25 mL) were taken from the jugular cannula at 0, 10, 15, 20, 30, 40, 60, 75, 90, 120 and 140 min; approximately the same volume of saline was used to clear the tubing. For intravenous administration, blood samples were taken at 0, 15, 30, 45, 60, 75, 90, 100, 120, 140, and 160 min. In the oral study, a blood sample (1.5 mL) was taken close to the last time point and was used for the assay of β-hydroxybutyrate and acetone. Blood samples were collected into heparinized tubes (Sarstedt microvette CB 300, Numbrecht, Germany) and centrifuged at 3000 *g* for 10 min. The resultant plasma was stored in −80°C until assay.

The rats were decapitated after the oral study and the liver removed. The large lobe was used to freshly prepare microsomes (Roberts et al 1995b); the microsomes were stored at −80°C until assay.

Seven rats each were used for the intravenous study (one receiving 25 mg kg^{-1} drug; three control rats; and four fasted rats) and the oral study (one receiving 50 mg kg^{-1} drug; three control rats; and four fasted rats) of chlorzoxazone. After the intravenous study, rats were re-fed for at least two days to wash-out any remaining chlorzoxazone before the oral study was conducted; the blood sampling schedule was the same as that used for the intravenous study. Another 11 animals were used only for the oral study (six for control group and five for fasted rats). The other experimental conditions were the same as mentioned above.

Analysis of chlorzoxazone and 6-hydroxychlorzoxazone

Plasma (50 μL) was added to a 1.5-mL microcentrifuge tube containing 60 μL buffer comprising: 40 μL 0.2 M acetate buffer pH 4.75; 10 μL 50 μg mL^{-1} pentoxifylline; and 10 μL β-glucuronidase (10 120 U mL^{-1} in 0.2% NaCl) (Lucas et al 1993). The plasma mixture was incubated at 37°C for 3 h, after which time 100 μL acetonitrile was added, vortexed and centrifuged at 15 300 *g* for 10 min. Supernatant (100 μL) was transferred to a second microfuge tube and 100 μL 10% trichloroacetic acid and 200 μL water were added. This tube was vortexed and centrifuged at 15 300 *g* for 10 min. A sample of supernatant (200 μL) was filtered through a Microcon YM-10 from Millipore Co. (Bedford, MA, USA) by centrifugation at 15 300 *g* for 25 min. Eluant (50 μL) was injected onto the HPLC. The Waters HPLC system consisted of a Model 600E pump, a Model 715 Ultra WISP autoinjector and a Model 486 Absorbance Detector set at 284 nm. Data acquisition and analysis was performed using the Dynamax system with the MacIntegrator II program (Varian, Walnut Creek, CA, USA). Separation was accomplished at 35°C on a Nova-Pak C-18 column (3.9 × 150 mm i.d.) by Waters (Milford, MA, USA). The mobile phase consisted of 86% water containing 0.15% ammonium acetate (pH 4.7 with glacial acetic) and 14% acetonitrile by pump mix with a flow rate of 1.1 mL min^{-1}. Retention times for 6-OHCZ, pentoxifylline (internal standard), and chlorzoxazone were at 3.5, 6.9 and 19.8 min, respectively. Linear calibration curves ($r > 0.99$) were obtained for both compounds in plasma over the concentration range from 1 to 50 μg mL^{-1}. For the two concentrations tested, 6.25 and 12.5 μg mL^{-1}, the intra-assay coefficients of variation were less than 5% and 6% for 6-OHCZ and chlorzoxazone, respectively; the inter-assay coefficients of variation were less than 6% and 7% for 6-OHCZ and chlorzoxazone, respectively. The limits of detection were estimated to be 0.05 μg mL^{-1} for both compounds.

Acetone analysis

Plasma (75 μL) was transferred to a gas-tight headspace vial. The samples were heated for 15 min at 70°C before analysis. The gas phase of the samples was analyzed with a gas chromatograph (Carlo Erba FTV 2350) as described by Ernstgård et al (1999), equipped with a headspace sampler (Hewlett Packard, HP7694) using a nonpolar capillary column (Poraplot Q, Chrompack 25 m, 0.53 mm i.d., 20-μm phase thickness), nitrogen as carrier gas and flame ionization detection. Injection and detection temperatures were 175 and 275° C, respectively. The column temperature was 130° C and the retention time of acetone was 3.7 min. Calibration standards of acetone (99% purity, Merck, Germany) were prepared using Millipore water. The detection limit was approximately 2 μmol L^{-1}.

Immunoblot analyses

Liver CYP2E1 level was determined by immunoblot analysis. After the microsomal protein (2.5 μg/lane) was resolved by gel electrophoresis (8% TG 1.0 gel by NOVEX), the protein was transferred to PVDF (polyvinylidene difluoride) membranes. Following incubation with blocking buffer (5 g non-fat dry milk and 20 mg NaN_3 in 100 mL 1X physiologic buffered saline (PBS) for 2 h, the membranes were then incubated overnight with primary antibody (1:1000 dilution in the same blocking buffer) (Roberts et al 1995a). After washing with wash solution (15 mL 5 M NaCl, 25 mL 1 M Tris-HCl (pH 7.4), 460 mL water, 3×10 min), they were incubated with secondary antibody (1:1000 dilution) in 5% non-fat dry milk in 1X PBS for 1 h. The membranes were washed three times for 10 min in wash solution and the membrane placed into 50 mL 0.01 M Tris-HCL (pH 7.4) containing 30 mg 3,3′-diaminobenzidine tetrahydrochloride dihydrate and 15 mg cobaltous chloride hexahydrate (Fluka, Buchs, Switzerland). The antigen protein bands were visualized by adding 15 μL 30% hydrogen peroxide. Immunoblots were quantitated by scanning them into Adobe Photoshop (Macintosh) and using NIH Image 1.61 to estimate the density of each band. In each blot, the average densitometric value from control rats was used as baseline, and the ratio of densitometric value from each individual band to the baseline was taken as the result, as described (Wan et al 2004).

Enzyme assays

Aniline hydroxylase activity was measured according to Roberts et al (1995b) except that the final aniline concentration was 1 mM. Erythromycin *N*-demethylase (ERNd) activity was determined as described by Wang et al (1997) with the following modifications: microsomes (0.5 mg mL^{-1}) were incubated with 500 μM erythromycin in 0.25 M Tris buffer (pH 7.4) with 6 mM $MgCl_2$, an NADPH generating system consisting of 1.2 mM $NADP^+$, 10 mM isocitrate, and 0.24 U isocitrate dehydrogenase; reactions were performed at 37°C for 15 min. Ethoxyresorufin *O*-dealkylase (EROD) and pentoxyresorufin *O*-dealkylase (PROD) activity were assayed as described by Burke et al (1985). Plasma β-hydroxybutyrate concentrations were measured using kits from Sigma (St Louis, MO, USA). Protein concentrations were determined by Pierce BCA protein assay kit (Rockford, IL, USA).

Pharmacokinetic analysis

To estimate the elimination rate of chlorzoxazone and 6-OHCZ from the body, the intravenous chlorzoxazone and 6-OHCZ data were sequentially fitted (Statistica 3.0b for the Macintosh) using equations 1 and 2, respectively. These equations were generated by Laplace transformation as described by Chen & Yang (1996). The molar concentration was used here only for fitting purposes.

$$C_p = (D/Vd_p)e^{-kt} \quad (1)$$

$$C_m = (k_f(D/Vd_m)/(k-k_m))[e^{-kmt} - e^{-kt}] \quad (2)$$

Where C_p (μmol mL^{-1}) was the plasma concentration of chlorzoxazone and C_m the concentration of the 6-OHCZ metabolite. Other parameters were dose (D, μmol kg^{-1}), apparent distribution volume (Vd_p, mL kg^{-1}), the rate of elimination of chlorzoxazone through all pathways (k, min^{-1}), the rate of formation of 6-OHCZ (k_f, min^{-1}), the rate constant for 6-OHCZ elimination (k_m, min^{-1}). Chen & Yang (1996) also defined k as being equal to $k_f + k_e$, where k_e is rate of elimination of chlorzoxazone through any other pathway.

In a non-compartmental analysis, the area under the plasma concentration–time curve (AUC_∞) was determined by the linear trapezoidal rule with extrapolation to infinity by use of a log–linear estimation of the terminal elimination constant (λ_z). The volume at steady-state (Vd_{ss}, mL kg^{-1}) of chlorzoxazone following intravenous administration was calculated by the equation: CL×MRT. The half-life (t½) for each compound was obtained from the ratio $0.693/\lambda_z$. The oral chlorzoxazone absorption fraction (F) was calculated according to equation 3:

$$F = (AUC_{(p.o.)}/Dose_{(p.o.)})/(AUC_{(i.v.)}/Dose_{(i.v.)}) \quad (3)$$

The oral clearance (CL/F, mL min^{-1}) of chlorzoxazone was calculated from $Dose_{(p.o.)}/AUC_{\infty(p.o.)}$.

Statistical analysis

Data are presented as mean ± s.d. The Student's *t*-test (Statistica 3.0b) was used to assess the significance of differences ($P < 0.05$) between the values in two treatment groups. To estimate the correlation of variables (aniline hydroxylase, EROD, ERNd, PROD activity and CYP2E1 level) to chlorzoxazone elimination parameters (1/t½), an analysis of covariance was used in a multiple analysis of variance test. A multiple regression was used to determine the correlation of above mentioned independent variables to 1/t½ for chlorzoxazone.

Results

Effects of fasting on chlorzoxazone metabolism

Intravenous study

Plots of average chlorzoxazone and 6-OHCZ plasma concentration following intravenous administration to control

and fasted rats are shown in Figure 1A and B, respectively. Parameters for the fit of equations 1 and 2 to chlorzoxazone and 6-OHCZ concentration are shown in Table 1.

Pharmacokinetic parameters for chlorzoxazone and 6-OHCZ calculated by non-compartmental analysis are shown in Table 2. The chlorzoxazone elimination rate constant k,

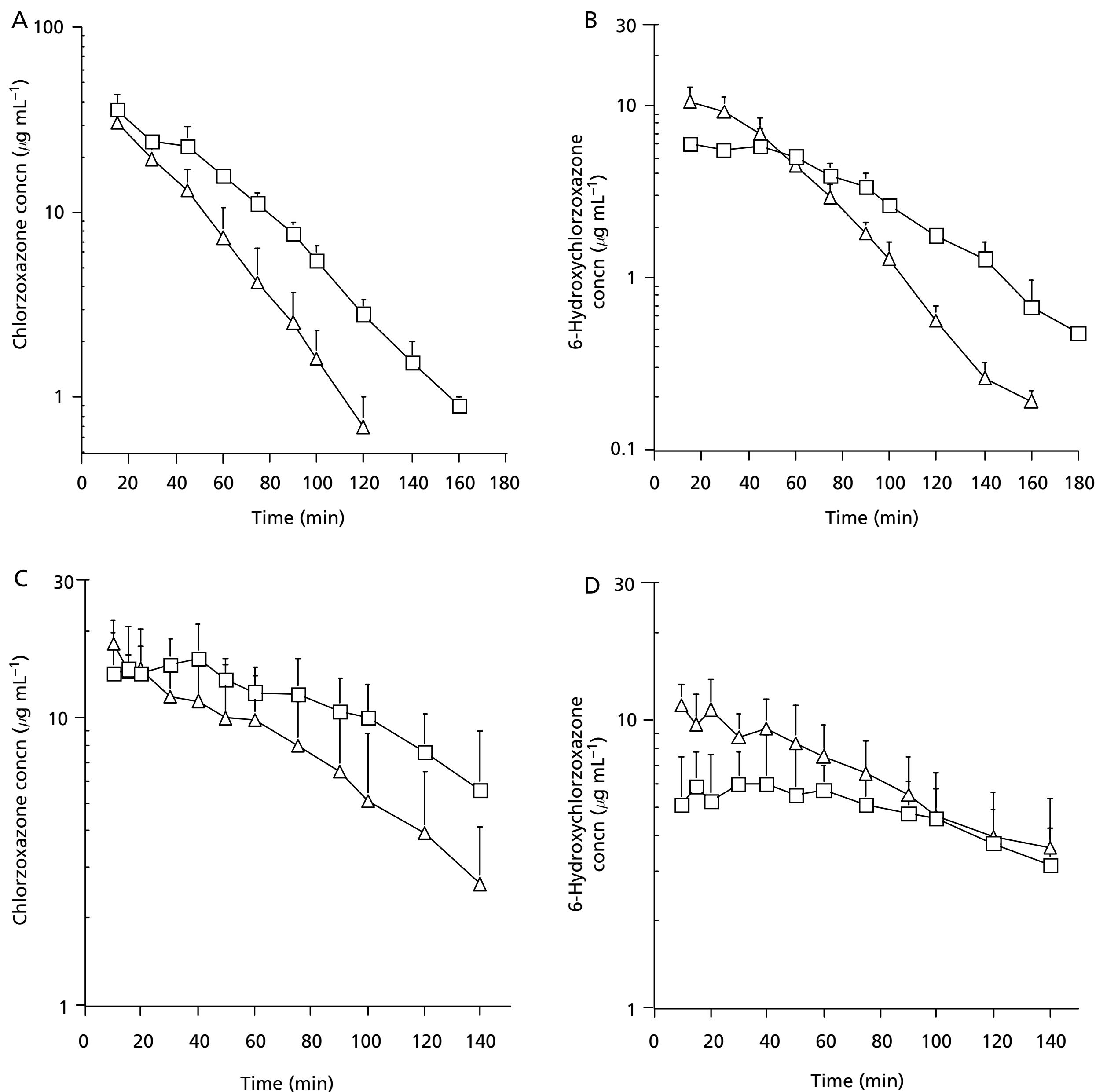

Figure 1 Time-dependent changes in plasma concentrations of chlorzoxazone and 6-hydroxychlorzoxazone (6-OHCZ) after intravenous and oral administrations of chlorzoxazone in control and fasted rats. Upper panels: plasma concentration vs time curve of chlorzoxazone (A) and 6-OHCZ (B) after a 25 mg kg^{-1} intravenous bolus dose of chlorzoxazone to control (square) and fasted (triangle) rats. Lower panels: plasma concentration vs time curve of chlorzoxazone (C) and 6-OHCZ (D) after a 50 mg kg^{-1} oral dose of chlorzoxazone; the symbols for groups are the same as above.

Table 1 Parameters obtained from the sequential fit of plasma chlorzoxazone and 6-hydroxychlorzoxazone concentrations, following a 25 mg kg^{-1} intravenous dose, to equations 1 and 2 (see Materials and Methods)

Treatment	$k \times 10^{-3}$ (min^{-1})	$k_f \times 10^{-3}$ (min^{-1})	$k_e \times 10^{-3}$ (min^{-1})	$k_m \times 10^{-3}$ (min^{-1})	Vd_p (mL kg^{-1})	Vd_m (mL kg^{-1})
Control (n = 3)	19.9 ± 1.9	9.9 ± 1.7	10.0 ± 0.06	52.6 ± 2.3	519 ± 117	423 ± 3
Fasted (n = 4)	31.8 ± 5.1[a]	22.2 ± 5.0[a]	9.6 ± 4.3	81.6 ± 5.6[a]	508 ± 65	384 ± 25

[a] $P < 0.05$ compared with control rats.

Table 2 Pharmacokinetic parameters of chlorzoxazone and 6-hydroxychlorzoxazone following a 25 mg kg^{-1} intravenous dose of chlorzoxazone to male Sprague-Dawley rats

Treatment	Chlorzoxazone						6-Hydroxychlorzoxazone		
	C_0 ($\mu g\ mL^{-1}$)	λ_z ($\times 10^3\ min^{-1}$)	t½ (min)	AUC_∞ ($\mu g\ mL^{-1}\ min^{-1}$)	CL ($mL\ min^{-1}\ kg^{-1}$)	Vd_{ss}* ($mL\ kg^{-1}$)	λ_z ($\times 10^3\ min^{-1}$)	t½ (min)	AUC_∞ ($\mu g\ mL^{-1}\ min^{-1}$)
Control (n = 3)	63.9 ± 16.9	31.4 ± 3.5	22.2 ± 2.6	2013 ± 325	12.6 ± 1.9	403 ± 71	19.9 ± 3.5	35.5 ± 6.7	592 ± 64
Fasted (n = 4)	51.7 ± 5.8	43.7 ± 4.9[a]	16.0 ± 1.7[a]	1196 ± 207[a]	21.3 ± 3.3[a]	491 ± 80	37.4 ± 3.5[a]	18.6 ± 1.9[a]	579 ± 107

[a] $P < 0.05$ compared with control rats. *$Vd_{ss} = Cl \times MRT$.

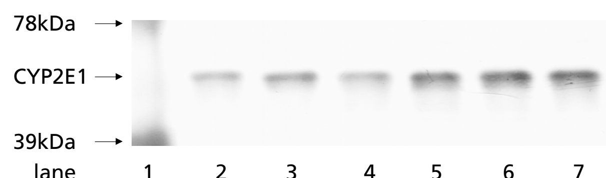

Figure 2 Immunoblot analysis of microsomal CYP2E1 contents in control and fasted rats. After the microsomal protein (2.5 μg/lane) was resolved by gel electrophoresis (8% TG 1.0 gel by NOVEX), the protein was transferred to PVDF membranes, and subjected to immunoblot analysis as detailed in the Materials and Methods section. Lane 1, molecular marker; lanes 2–4, control; lanes 5–7, fasted rats.

determined by fitting equation 1, was increased 1.6-times by fasting ($P<0.05$); λ_z was increased 1.5-fold. This was in line with the immunoblot result, which showed fasting increased the CYP2E1 level 1.76-fold (Figure 2, Table 3). The rate constants for 6-OHCZ formation and elimination, k_f and k_m, were also increased by fasting ($P<0.05$). The excellence of curve fitting was confirmed by visual inspection of the plot, and the values of k and k_f in control rats were similar to those reported by Chen & Yang (1996). The Vd_{ss} of chlorzoxazone (mL kg^{-1}) was similar in the two groups; there was no difference between fitted Vd_p and Vd_d determined by non-compartmental analysis.

The AUC of 6-OHCZ was unchanged by fasting even though the 6-OHCZ concentration and the 6-OHCZ/chlorzoxazone ratio were 2–3 times higher in fasted rats compared with those in control rats from 15 to 75 min (see Figure 1B). This should have been caused by increased production rate and elimination rate of 6-OHCZ by fasting. Although we used a small number of animals in each group, the very small standard deviation (Figure 1) indicated that the results were consistently reproducible and we therefore were able to find the significant difference.

Oral study

Plots of average chlorzoxazone and 6-OHCZ plasma concentration following oral administration to control and fasted rats are shown in Figure 1C and D, respectively. Fasting significantly decreased the AUC and increased the plasma clearance of chlorzoxazone (Table 4, Figure 1C). It appeared that the value of F (Table 4) in fasted rats was higher compared with control rats but the difference was not significant. The CL/F of chlorzoxazone was increased 1.6-times by fasting ($P<0.05$); t½ was decreased 1.6-fold ($P<0.05$). This was in line with the immunoblot result which showed fasting increased the CYP2E1 level 1.76-fold. The t½ of 6-OHCZ was faster in fasted rats, which was consistent with the intravenous study. The 6-OHCZ concentration and the ratio of 6-OHCZ/chlorzoxazone at 15 min were increased in fasted animals.

Effect of fasting on liver CYP2E1 level and microsomal activity

Liver CYP2E1 levels and microsomal enzyme activity are shown in Table 3 and Figure 2. Fasting increased the activity of aniline hydroxylase and PROD, and the immunoreactive CYP2E1 level.

Biochemical changes following fasting treatment

Fasting for 24 h increased plasma β-hydroxybutyrate concentrations significantly (from 0.17 ± 0.10 to 1.01 ± 0.29 mM). Fasting increased acetone concentration 2.6-fold (from 223 ± 126 to 591 ± 209 μM). As shown in Figure 3, the correlation between liver CYP2E1 level and activity and plasma acetone concentration was significant ($r=0.76$, $P<0.001$ and $r=0.68$, $P<0.001$, respectively), although the correlation between 1/t½ and plasma acetone concentration was somewhat weak despite its significance ($r=0.59$, $P<0.05$). Plasma β-hydroxybutyrate concentration significantly correlated with the liver CYP2E1 level, activity and 1/t½ ($r=0.87$, $P<0.001$; $r=0.66$, $P<0.001$; and $r=0.633$, $P<0.005$, respectively; Figure 3, right panel). The correlation between the plasma β-hydroxybutyrate and acetone concentration was also significant ($r=0.79$, $P<0.001$).

Analysis of covariance

The value of 1/t½ for chlorzoxazone was significantly linearly correlated with CYP2E1 level ($r=0.698$, $P<0.05$, Figure 4, left panel) and liver aniline hydroxylase activity ($r=0.665$, $P<0.05$, Figure 4, right panel). The value of CL/F for chlorzoxazone was also significantly correlated with CYP2E1 level ($r=0.63$, $P<0.05$). In the multiple analysis of variance analysis using 1/t½ as a dependent variable and grouped for two treatments, five variables were selected as simultaneous covariates: aniline hyroxylase, EROD, ERNd, PROD activity and CYP2E1 level. The value of 1/t½ was found to significantly correlate to CYP2E1 level ($t=2.56$, $P<0.05$) and aniline hyroxylase enzyme activity ($t=2.66$, $P<0.05$). None of the remaining variables was significantly correlated to 1/t½ ($P>0.2$). After using aniline hyroxylase activity or CYP2E1

Table 3 Liver CYP2E1 level and microsomal activity in control and fasted male Sprague-Dawley rats

Treatment	CYP2E1	Aniline hydroxylase (nmol min^{-1} mg^{-1})	Ethoxyresorufin *O*-dealkylase (nmol min^{-1} mg^{-1})	Pentoxyresorufin *O*-dealkylase (pmol min^{-1} mg^{-1})	Erythromycin *N*-demethylase (nmol min^{-1} mg^{-1})
Control (n = 9)	1.00 ± 0.22	0.42 ± 0.07	0.47 ± 0.13	39 ± 8	2.01 ± 0.13
Fasted (n = 9)	1.76 ± 0.33[a]	0.62 ± 0.13[a]	0.42 ± 0.14	52 ± 8[a]	1.96 ± 0.16

[a]$P<0.05$ compared with control rats.

Table 4 Pharmacokinetic parameters of chlorzoxazone and 6-hydroxychlorzoxazone (6-OHCZ) following a 50 mg kg^{-1} oral dose of chlorzoxazone to male Sprague-Dawley rats

Treatment	Chlorzoxazone					6-Hydroxychlorzoxazone				Ratio(15)†
	λ_z (× 10^3 min^{-1})	t½ (min)	CL/F* (mL min^{-1} kg^{-1})	AUC_∞* (μg mL^{-1} min^{-1})	F‡	λz (× 10^3 min^{-1})	t½ (min)	AUC_∞* (μg mL^{-1} min^{-1})	6-OHCZ(15)# (μg mL^{-1})	
Control (n = 9)	13.1 ± 3.8	57.8 ± 19.4	10.5 ± 2.9	1946 ± 547	0.46 ± 0.06	10.1 ± 3.2	74.3 ± 20.4	1028 ± 276	5.7 ± 1.8	0.37 ± 0.09
Fasted (n = 9)	21.5 ± 9.[a]	35.9 ± 10.8[a]	16.4 ± 6.9[a]	1272 ± 568[a]	0.59 ± 0.12	15.1 ± 6.2	51.5 ± 16.7[a]	1136 ± 429	9.6 ± 2.9[a]	0.72 ± 0.26[a]

*Due to incomplete early time curves, this parameter could not be calculated for all animals, therefore, n = 7 for control, n = 7 for fasted rats. ‡Oral bioavailability, n = 3 for control, n = 4 for fasted. #6-OHCZ(15) indicates the value of 6-OHCZ concentration at 15 min. †Ratio(15) indicates the ratio of 6-OHCZ to chlorzoxazone at 15 min. [a]$P < 0.05$ compared with control rats.

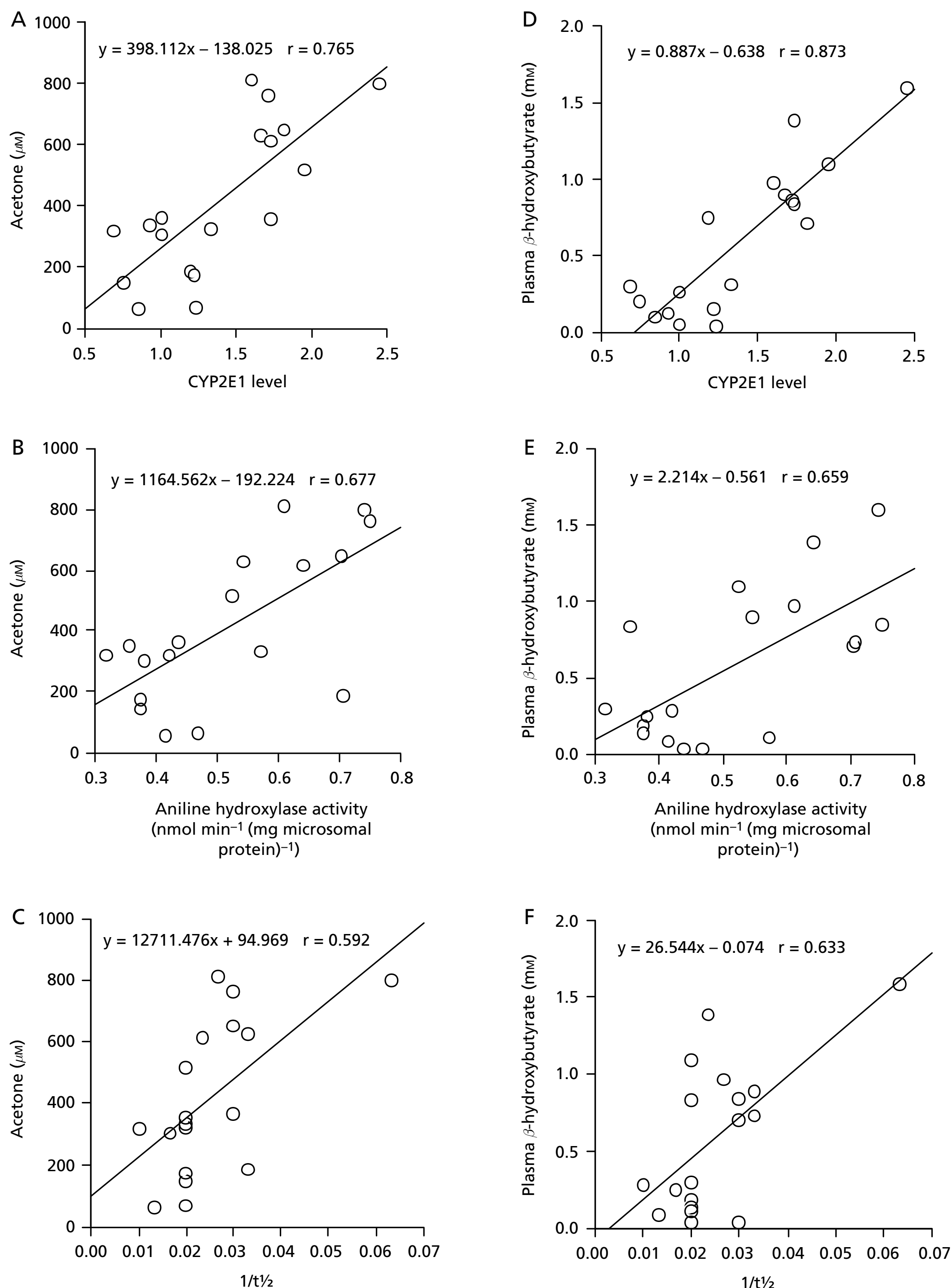

Figure 3 Correlation between plasma acetone or β-hydroxybutyrate and the levels of CYP2E1 contents, activity, or chlorzoxazone t½. Left panels: correlation between plasma acetone concentration and liver CYP2E1 level (A), liver CYP2E1 activity (B) and chlorzoxazone t½ (C) in the oral study. Right panels: correlation between plasma β-hydroxybutyrate concentration and liver CYP2E1 level (D), liver CYP2E1 activity (E) and chlorzoxazone t½ (F) in the oral study.

level alone for covariate, the difference in adjusted means between groups became insignificant ($P > 0.6$). Compatible with covariate analysis, when multiple regression was used with aniline hyroxylase, EROD, ERNd, PROD activity and CYP2E1 level as independents, aniline hyroxylase and CYP2E1 were found to significantly correlate with 1/t½

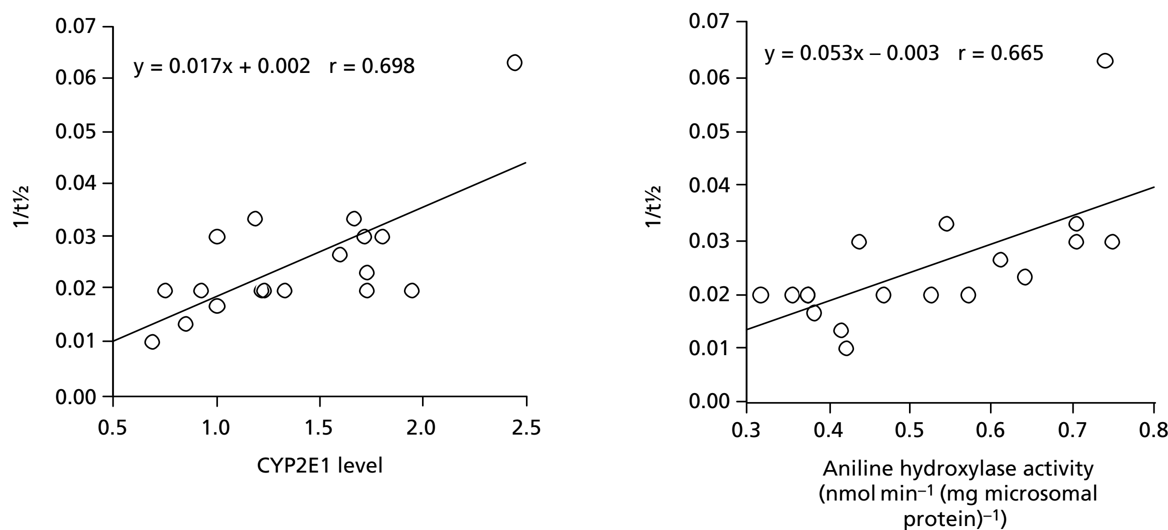

Figure 4 Correlation between chlorzoxazone t½ and the levels of CYP2E1 content or its activity. Left panel: correlation between chlorzoxazone t½ and liver CYP2E1 level. Right panel: correlation between chlorzoxazone t½ and liver CYP2E1 activity in the oral study.

($P < 0.05$), but none of the remaining variables were significantly correlated to 1/t½ ($P > 0.15$).

Discussion

We have investigated the effects of fasting on the pharmacokinetics of chlorzoxazone following oral and intravenous administration. Although the intravenous method was more suitable for determination of the terminal elimination rate, we investigated the rate of chlorzoxazone elimination after oral administration to compare the chlorzoxazone pharmacokinetic parameters with the liver CYP2E1 level or activity, since oral administration is more frequently used, especially in man. We found that fasting rats for 24 h significantly increased chlorzoxazone elimination as judged by t½ and CL/F in both studies, where the changes in in-vivo chlorzoxazone metabolism positively corresponded with the level of CYP2E1 determined by immunoblot analysis. This was opposite to the finding of O'Shea et al (1994) who reported that the value of t½ was increased and the oral systemic clearance of chlorzoxazone was reduced in 38-h fasted men. We also found that the induced clearance of chlorzoxazone was associated with the increased production rate of 6-OHCZ (see Table 4, last column). The observed difference in chlorzoxazone t½ between the oral and intravenous study was probably caused by the absorption rate in the oral study.

Our different finding in rats indicated that interspecies differences might exist in the fasting-induced change in chlorzoxazone metabolism. In other fasting studies, changes in plasma β-hydroxybutyrate concentrations have been used as a marker of the extent of fasting (Yun et al 1992; O'Shea et al 1994). In our rat study, β-hydroxybutyrate concentrations increased 6-fold. In the O'Shea et al (1994) study, β-hydroxybutyrate concentrations were increased 23-fold following 38-h fasting (20 ± 14 to 463 ± 212 μM). Based on this marker and high correlation between blood β-hydroxybutyrate and acetone concentrations, it would be conceivable that liver CYP2E1 could have been induced in man since acetone is known to increase CYP2E1 level (Song et al 1989), or CYP2E1 was likely induced by endogenously produced β-hydroxybutyrate and/or its closely related compound acetoacetate (Yun et al 1992). Since insulin concentration is known to play a role in the down regulation of CYP2E1 and it can be decreased by fasting (Woodcroft & Novak 1997), it could be that insulin concentrations are another key determinant of whether CYP2E1 induction is likely to occur.

Although fasting produces more acetone, acetone is a competitive inhibitor of CYP2E1 (Yoo et al 1987; Bondoc et al 1999). In our study, control animals had a mean acetone concentration of 223 μM and fasted animals had a mean concentration of 591 μM. But much lower concentrations of acetone appeared to inhibit chlorzoxazone metabolism in man. When chlorzoxazone was administered to volunteers concurrently inhaling acetone (Ernstgård et al 1999), chlorzoxazone concentrations at 3 h were 50.6 μM compared with 26.8 μM for control subjects ($P = 0.06$). The 6-OHCZ/chlorzoxazone ratios were lower than that for control subjects; peak acetone concentrations at 2–3 h were approximately 250 μM compared with 20 μM in baseline. Since it has been reported that blood acetone concentrations can be as high as 760 μM following three days of fasting (Reichard et al 1979), high concentration acetone inhibition of chlorzoxazone metabolism cannot be excluded as a possible mechanism behind the reduced clearance observed by O'Shea et al. (1994). Based on our rat data that the plasma acetone concentration was positively correlated with 1/t½ and the liver CYP2E1 activity, we believe that the inhibition of chlorzoxazone metabolism by acetone should be minor due to relatively low acetone concentrations in the fasted rats. However, we cannot exclude a possibility of competitive inhibition of chlorzoxazone 6-hydroxylation by a much higher acetone concentration

produced after an extended period of fasting, although CYP2E1 may be continuously induced as well (Hong et al 1987).

The dose we chose was similar to that used by other scientists (Chen & Yang 1996) who reported that chlorzoxazone 6-hydroxylation in-vivo could readily reflect CYP2E1 induction or inhibition. As the apparent K_m of chlorzoxazone 6-hydroxylation is 40–70 μM in man (Lucas et al 1999), the catalytic activity of CYP2E1 might be reflected more accurately at these plasma concentrations (assuming a plasma/liver partition coefficient of 1). The maximal plasma concentrations in the study of O'Shea et al. (1994), who used a 250 mg chlorzoxazone dose, were less than 35 μM. In addition, CYP2E1 in rats and man is known to be regulated in a similar manner. For instance, rat CYP2E1 was shown to be induced by alcohol and in fasting, diabetic, or obese condition (Hong et al 1987; Song et al 1987; Raucy et al 1991). Human CYP2E1 was thought to be induced in these conditions as well (Lucas et al 1995). In this regard, it might be of interest that a higher dose of chlorzoxazone such as 500 mg (Lucas et al 1999) or higher given to fasted volunteers would show different results.

Our chlorzoxazone peak concentrations in control rats were similar to those published by Kaneko et al (1990), who used a 25 mg kg^{-1} dose also. However, our control chlorzoxazone AUC was a little higher than their results of 1629 μg mL^{-1} min^{-1}. This difference might have resulted from a different fasting period before chlorzoxazone administration. Kaneko et al (1990) fasted the rats only overnight (>10 h) before dosing, which would probably only slightly induce CYP2E1. Our fitted k and k_f values were similar to those reported by Chen & Yang (1996), although they used a slightly larger dose (approximately 25.4 mg kg^{-1}). Fasting significantly increased the 6-OHCZ elimination in rats based on the fitted k_m of 6-OHCZ, suggesting a possible increase of renal clearance of conjugated 6-OHCZ since 6-OHCZ is excreted in the urine primarily as a glucuronide conjugate (Conney & Burns 1960). This result suggested that caution must be taken when 6-OHCZ/chlorzoxazone is used at certain time points, such as 2 h after dosing, as an index for phenotyping CYP2E1 in man with certain physiological conditions, such as fasting and diabetes with possibly altered 6-OHCZ elimination, although this index has been used successfully in many other studies (Lucas et al 1999; Ernstgård et al 1999). When chlorzoxazone is used as a tool to phenotype CYP2E1 in volunteers with starvation or diabetes, it might be appropriate to use CL and t½ of chlorzoxazone as indexes following a larger oral dose such as 500 or 750 mg.

For orally dosed animals, the value of CL/F could not be calculated for four rats because an accurate AUC could not be determined due to technical difficulties at early sampling times. Due to smaller sample size and F varied individually, it was unacceptable to use CL/F for correlation analysis. In the multiple analysis of variance and regression analysis, we used the reciprocal of t½ of chlorzoxazone (1/t½) as the dependent variable because 1/t½ is linearly related to CL via the following relationship: t½ = 0.693 F·V/CL. We were able to determine the value of CL/F for all animals, and so this value was used instead. After using multiple analysis of variance covariate analysis and regression analysis, the value of 1/t½ was found to significantly correlate with liver CYP2E1 level and aniline hydroxylase (CYP2E1) activity. Therefore, our results suggested that CYP2E1 was the predominant enzyme in catalysing chlorzoxazone biotransformation in-vivo. Our results were consistent with the findings of others (Yamazaki et al 1995; Chen & Yang 1996; Lucas et al 1999), who demonstrated that the role of other P450s in chlorzoxazone metabolism in-vivo was minor. Specifically, Lucas et al (1999) reported that cigarette smoking, a CYP1A inducer, and grapefruit juice, a CYP3A inhibitor, failed to modify the chlorzoxazone metabolic ratio in volunteers. They estimated that the contribution of CYP1A and CYP3A to chlorzoxazone 6-hydroxylation activity was approximately 0.5% and 2.5% of total activity, respectively. In contrast, other scientists suggested that CYP3A and CYP1A might play significant roles in chlorzoxazone metabolism in man, despite being less important as compared with CYP2E1 (Ono et al 1995; Shimada et al 1999). It was found that CYP4A was induced by starvation (Kroetz et al 1998), but it was possibly not involved in chlorzoxazone 6-hydroxylation (Hanioka et al 1998). Shimada et al (1999) reported that CYP2B6 had a considerable activity in chlorzoxazone 6-hydroxylation in-vitro, however, it should play a relatively minor role in-vivo because of its low expression in the liver.

In agreement with the findings of Fry et al (1999) and Brown et al (1995), our data showed that CYP2B activity was increased and CYP1A activity was unchanged by fasting. However, in contrast with the finding by Fry et al (1999), we did not observe an increase in CYP3A activity. The reason for this discrepancy was not clear.

Conclusions

Fasting for 24 h induced chlorzoxazone metabolism in male Sprague-Dawley rats. This was in contrast to the decreased metabolism of chlorzoxazone reported for 38-h fasted men. The in-vivo chlorzoxazone elimination parameter 1/t½ was significantly correlated with liver CYP2E1 level, and the contribution of other P450s to chlorzoxazone metabolism might be minor in the concentration range tested in the rats of this study.

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