DEVELOPMENT OF A NON-HIGH PRESSURE LIQUID CHROMATOGRAPHY ASSAY TO DETERMINE [14C]CHLORZOXAZONE 6-HYDROXYLASE (CYP2E1) ACTIVITY IN HUMAN LIVER MICROSOMES

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ABSTRACT:

The activity of liver microsomal CYP2E1 is commonly measured as the rate of 5-chloro-2-benzoxazolone (chlorzoxazone) 6-hydroxylation, which requires separation of 6-hydroxychlorzoxazone and chlorzoxazone by high pressure liquid chromatography (HPLC). In the present study, we describe a solvent extraction (non-HPLC) assay for measuring CYP2E1 activity, based on the 6-hydroxylation of [14C]chlorzoxazone. When [14C]chlorzoxazone was incubated with human or rat liver microsomes in the presence of NADPH, the major product formed was 6-[14C]hydroxychlorzoxazone. Unreacted [14C]chlorzoxazone was quantitatively extracted from the incubation mixture with dichloromethane under conditions that resulted in ~45% extraction of 6-[14C]hydroxychlorzoxazone. The amount of 6-[14C]hydroxychlorzoxazone remaining in the aqueous incubation mixture (~55% of the total amount formed) was quantified by liquid scintillation spectrometry. The limit of detection for this assay was 100 pmol of 6-[14C]hydroxychlorzoxazone. The solvent extraction procedure was validated by comparing the rates of formation of 6-[14C]hydroxychlorzoxazone with those determined by HPLC under a variety of experimental conditions. The close correspondence between the two analytical methods suggests that the extraction procedure for measuring 6-[14C]hydroxychlorzoxazone provides a simple, sensitive, and rapid alternative to the HPLC procedure for measuring CYP2E1 activity. In rats, the assay is not specific for CYP2E1 because CYP1A1 also catalyzes the 6-hydroxylation of chlorzoxazone. Recombinant human CYP1A1 also catalyzed the 6-hydroxylation of chlorzoxazone (at ¼ the rate of CYP2E1), although CYP1A1 is not expressed in human liver microsomes. The non-HPLC assay was used to investigate the postulated role of CYP1A2 in the 6-hydroxylation of chlorzoxazone by human liver microsomes. Recombinant CYP1A2 did not catalyze the 6-hydroxylation of chlorzoxazone, and studies with 1-[3,4-dimethoxyphenyl]methyl]-6,7-dimethoxyisoquinoline, which inhibits CYP1A2 but not CYP2E1, indicated that, in human liver microsomes, the 6-hydroxylation of chlorzoxazone is catalyzed by CYP2E1 with little or no contribution from CYP1A2 enzymes over a wide range of substrate concentrations.

CYP2E1 is one of several cytochrome P450 enzymes in human liver (Koop, 1992; Koop and Tierney, 1990; Parkinson, 1996; Yang et al., 1990). The enzyme is also expressed in certain extraparenchymal tissues, such as kidney, lung, and lymphocytes, and is inducible by ethanol, isoniazid, pyrazole, pyridine, and ketogenic disorders, such as fasting and uncontrolled diabetes (Koop, 1992; Koop and Tierney, 1990; Parkinson, 1996; Perrot et al., 1989; Song et al., 1990; Yang et al., 1990). Substrates for CYP2E1 include simple alcohols, nitrosamines, aliphatic chlorohydrocarbons, such as carbon tetrachloride, and small aromatic hydrocarbons, such as benzene and aniline (Eckstrom et al., 1989; Guengerich et al., 1991; Parkinson, 1996; Yang et al., 1990). Compared with some forms of cytochrome P450, CYP2E1 metabolizes few drugs, although it can activate dapsone to its hydroxylamine metabolite (Mitra et al., 1995), catalyze the N1- and N7-demethylation of caffeine to theobromine and theophylline (Gu et al., 1992; Tassaneeyakul et al., 1994), activate acetonaminophen to the hepatotoxic metabolite, N-acetylbenzoquinoneimine (Patten et al., 1993; Raucy et al., 1989), and catalyze the dehalogenation of several chlorofluorocarbons (Herbst et al., 1994; Surbrook and Olson, 1992) and volatile anesthetics, such as halothane (Kharasch and Thummel, 1993; Madan and Parkinson, 1996; Thummel et al., 1993).

In human liver microsomes, CYP2E1 activity can be conveniently measured as 5-chloro-2-benzoxazolone (chlorzoxazone) 6-hydroxylation activity (Gillam et al., 1994; Peter et al., 1990; Tassaneeyakul et al., 1993; Yamazaki et al., 1995) and 4-nitrophenol hydroxylation activity (Tassaneeyakul et al., 1993a, 1993b). The 6-hydroxylation of chlorzoxazone can also be catalyzed by CYP1A1 (Carriere et al., 1993; Yamazaki et al., 1995), but this enzyme is rarely if ever expressed in human liver (Murray et al., 1993; Schweikl et al., 1993). Chlorzoxazone is an FDA-approved muscle relaxant (Paraflex), and

1 Abbreviations used are: chlorzoxazone, 5-chloro-2-benzoxazolone; papavere, 1-[3,4-dimethoxyphenyl]methyl]-6,7-dimethoxyisoquinoline; zoxazolamine, 5-chloro-2-benzoxazolamine; HPLC, high pressure liquid chromatography.
the urinary excretion of 6-hydroxychlorzoxazone and the plasma ratio of 6-hydroxychlorzoxazone to chlorzoxazone have been used as non-invasive in vivo probes of CYP2E1 (Dreischub et al., 1995; Girre et al., 1994; Kharasch et al., 1993; Kim et al., 1995; O’Shea et al., 1994). The 4-nitrophenol hydroxylase assay involves the spectrophotometric determination of 4-nitrocatechol (Tassaneeyakul et al., 1993a, 1993b). Although 4-nitrophenol hydroxylation provides a non-HPLC assay of CYP2E1 activity, the method is not very sensitive. The sensitivity of the 4-nitrophenol hydroxylase assay cannot be increased simply by increasing the concentration of substrate because other P450 enzymes, such as CYP2A6, contribute to this reaction when the concentration of 4-nitrophenol exceeds 200 μM (Draper et al., 1996a). On the other hand, chlorzoxazone 6-hydroxylation, although relatively specific for human CYP2E1, requires HPLC analysis, and is time consuming. In the present study, we describe a solvent extraction procedure to measure the rate of 6-hydroxylation of [14C]-chlorzoxazone. This procedure provides a rapid, sensitive, non-HPLC method for measuring CYP2E1 activity in human liver microsomes.

Materials and Methods

Chemicals and Reagents. Chlorzoxazone, 4-methylpyrazole, papaverine, and all cofactors were purchased from Sigma. All solvents were purchased from Fisher Scientific. [2-14C]Chlorzoxazone (specific activity, 55 Ci/mol) was purchased from Amersham International. 6-Hydroxychlorzoxazone was a gift from F. Peter Guengerich (Vanderbilt University, Nashville, TN) and Dr. John Lipscomb (Wright Patterson Air Force Base, OH). 7-Ethoxyresorufin and resorufin were purchased from Molecular Probes Inc. Microsomes prepared from B-lymphoblastoid cell line transfected with cDNAs for human P450 were purchased from Amersham International. 6-Hydroxychlorzoxazone was a gift from XenoTech, L.L.C. (Kansas City, KS). All other solvents were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ).

Preparation and Isolation of 6-[14C]Hydroxychlorzoxazone. 6-[14C]Hydroxychlorzoxazone was prepared by reverse phase HPLC, based on the method of Peter et al. (1990) with modifications described by Pearce et al. (1996), whereas the 6-hydroxylation of [14C]chlorzoxazone was measured by the new solvent extraction method. Human or rat liver microsomes (0.4 mg) were incubated at 37±1°C in 1.0 ml incubation mixtures (final volume) containing potassium phosphate buffer (50 mM, pH 7.4), MgCl2 (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), and glucose-6-phosphate dehydrogenase (1 unit/ml) and chlorzoxazone (10–50 μM with or without 0.5 μCi of [14C]chlorzoxazone) at the final concentrations indicated. Substrate solutions were prepared fresh for each experiment. Chlorzoxazone was added to each 1.0-ml incubation mixture in 20 μl of 60 mM potassium hydroxide. 14C-Chlorzoxazone was supplied by the manufacturer as an ethanolic solution. Because ethanol is a CYP2E1 substrate, the solvent was evaporated, and the residue of 14C-Chlorzoxazone was dissolved in 60 mM potassium hydroxide to give a final concentration of 0.5 μCi/incubation, 20 μl of which was added to designated incubation mixtures. The chemical inhibitors, 4-methylpyrazole and papaverine, were dissolved in water for addition to the incubation mixtures. All reactions were started by addition of the NADPH-generating system.

HPLC Analysis. Incubations containing unlabeled chlorzoxazone were stopped after 0–60 min with 200 μl of 30% perchloric acid containing 500 μM zoxazolamine (internal standard). Precipitated protein was removed by centrifugation (2000g for 10 min), and an aliquot (up to 100 μl) of the clear supernatant fraction was analyzed with a binary HPLC system (Shimadzu Scientific Instruments, Columbia, MD). Chlorzoxazone, 6-hydroxychlorzoxazone, and zoxazolamine (internal standard) were resolved by binary gradient HPLC on a Zorbax ODS reversed phase octadecylsilylam column (5-μm particle size, 15 cm × 4.6 mm i.d. from MAC-MOD Analytical, Chadds Ford, PA) preceded by a Supelcoil LC-18 guard column (40-μm particle size, 2 cm × 4.6 mm, i.d.) (Supelco, Bellefonte, PA). Mobile phase A was an 87.5:12.5 (v/v) mixture of 20 mM sodium perchlorate (pH 2.5) and acetonitrile, whereas mobile phase B was a 70:30 mixture of the same components. The total flow rate was 2 ml/min, and the column temperature was 30±1°C. The solvent program was as follows: 100% mobile phase A from 0 to 4 min, a step gradient to 100% B at 4 min, 100% mobile phase B maintained from 4 to 7.5 min, a step gradient to 100% A at 7.5 min, followed by re-equilibration with 100% mobile phase A from 7.5 to 12.0 min. Total analysis time was 15 min per sample. The retention times of 6-hydroxychlorzoxazone, zoxazolamine, and chlorzoxazone were approximately 4, 7.5, and 8.9 min, respectively. Metabolites were monitored at 287 nm with a variable wavelength UV detector. The amount of 6-hydroxychlorzoxazone was quantified by comparison of its peak areas with that of authentic standard.

Differential Extraction. The 6-hydroxylation of [14C]chlorzoxazone was carried out in 1-ml incubation reactions (as described above), which were stopped with 6 ml of dichloromethane. To increase the aqueous solubility of the metabolite, the pH of the aqueous phase was adjusted to 8.5 by the addition of 1 ml of EDTA (20 mM, pH 9.6), bringing the volume of aqueous phase to 2 ml. The tubes were vigorously mixed on a batch vortexer, and the aqueous and organic phases were separated by centrifugation (2000g for 10 min). A 1-ml aliquot of the aqueous phase (i.e. 50%) was transferred to another tube, and traces of the substrate, chlorzoxazone, were extracted with an additional 6 ml of dichloromethane; the two phases were then separated by centrifugation (2000g for 10 min). A 0.5-ml aliquot of the aqueous phase, corresponding to 25% of the initial aqueous phase, was transferred to a scintillation vial containing 5 ml of biodegradable scintillation cocktail (Econo-Safe, Research Products International, Mount Prospect, IL), and the amount of radioactivity was determined with a Beckman LS6500 multi-purpose scintillation counter. Zero-time incubations served as blanks. Blanks spiked with 2.5, 5, and 10 nmol of 6-[14C]Chlorzoxazone (60 mCi/mol; prepared as described below) served as standards. To determine the amount of radioactivity added to each incubation mixture, 0.5 μCi of [14C]cholorzoxazone was added directly to 5 ml of scintillation cocktail. All samples and standards were incubated in duplicate or triplicate. The experimental conditions were designed such that no more than 15% of the substrate was converted to 6-hydroxychlorzoxazone, which is the major detectable metabolite formed by human and rat liver microsomes under these experimental conditions (fig. 1).

Preparation and Isolation of 6-[14C]Hydroxychlorzoxazone. 6-[14C]Hydroxychlorzoxazone is not commercially available, so this radioactive metabolite was generated from [14C]chlorzoxazone and purified by HPLC. Liver microsomes from streptozotocin-treated rats (1 mg) were incubated with [14C]chlorzoxazone (~2 Ci/mol, 250 μM) essentially as described above. After a 60-min incubation, each 1-ml incubation reaction was stopped with 4 ml of dichloromethane-acetone (4:1, v/v). The aqueous (upper) layer was removed and extracted twice more with 4 ml of dichloromethane-acetone (4:1, v/v). The organic phase was evaporated to dryness in a Speed-Vac concentrator. The residue was reconstituted in a 250-μl mobile phase, and 6-[14C]Chlorzoxazone was purified by HPLC as described above. The purified metabolite was diluted ~35-fold with nonradioactive 6-hydroxychlorzoxazone to give a final specific activity of ~60 mCi/mol.

7-Ethoxyresorufin O-Dealkylation. The O-dealkylation of 7-ethoxyresorfin was measured by the fluorimetric method of Burke and Mayer (1974) with minor modifications (Dutton and Parkinson, 1989; Pearce et al., 1996). Human
Liver microsomes (0.1 mg) were incubated at 37°C in 1-ml incubation mixtures containing potassium phosphate buffer (100 mM, pH 7.4), MgCl₂ (3 mM), EDTA (1 mM), NADP (1 mM), glucose 6-phosphate (5 mM), glucose 6-phosphate dehydrogenase (1 Unit/ml), and 7-ethoxyresorufin (0.125 to 5.0 μM) at the final concentrations indicated. 7-Ethoxyresorufin was added to each incubation in 4 μl of dimethyl sulfoxide. Reactions were started by addition of the NADPH-generating system and were stopped after 2 min by the addition of 2 ml of ice-cold acetone. Precipitated protein was removed by centrifugation (2000g for 10 min). The amount of resorufin in the clear supernatant fraction was determined fluorimetrically (λ exc = 535 nm, λ em = 585 nm) with a Shimadzu RF 540 spectrofluorometer. Zero-time incubations served as blanks, and blanks spiked with 20–1000 pmol of resorufin served as standards. All samples and standards were incubated in duplicate.

**Analysis of Kinetic Constants.** The apparent kinetic constants (V max, K m, and K i) were determined by an Enzyme Kinetics program from Trinity Software (Campton, NH, version 1.4.1), which weights data toward the higher concentration of the substrate and/or low concentrations of inhibitor (weighting factor = 4).

**Results**

Solvent extraction procedures have been used to measure the P450-dependent metabolism of several substrates, ranging from highly hydrophobic compounds, such as benzpyrene (Nesnow et al., 1977), to relatively polar compounds, such as lauric acid (Giera and van Lier, 1991). These procedures exploit differences in the aqueous solubility of the substrate and its metabolite(s), such that the more polar substrate can be extracted with organic solvent under conditions that leave most or all of the metabolite(s) in the aqueous medium.

Based on HPLC analysis, 6-hydroxychlorzoxazone is the product formed when chlorzoxazone is incubated with human or rat liver microsomes in the presence of NADPH (fig. 1). Based on their chromatographic behavior, 6-hydroxychlorzoxazone was judged to be considerably more polar than chlorzoxazone, raising the possibility that these two compounds could be separated by a solvent extraction procedure. To this end, several organic solvents were examined for their ability to differentially extract chlorzoxazone and 6-hydroxychlorzoxazone from incubation mixtures (buffered to pH 7.4). Dichloromethane was more effective than the other organic solvents examined (ethylacetate, toluene, and chloroform) at differentially extracting chlorzoxazone and 6-hydroxychlorzoxazone (results not shown). However, although 98–99% of chlorzoxazone could be extracted from a 1-ml incubation mixture with 6 ml of dichloromethane, this was associated with a 50–60% loss of 6-hydroxychlorzoxazone.

For the extraction procedure, each 1.0-ml incubation mixture contained 0.5 μg of [14C]chlorzoxazone (~1.1 × 10⁶ dpm). In zero-time (blank) incubations, the amount of radioactivity remaining in the aqueous phase (pH 8.5) following two extractions with dichloromethane was 230 ± 20 dpm (mean ± SD of four independent experiments). This represents 0.02% of the total amount of radioactivity, indicating 99.98% of the added [14C]chlorzoxazone was extracted.
with dichloromethane. In incubated samples (i.e., in samples containing the metabolite 6-[14C]hydroxychlorzoxazone), the amount of radioactivity remaining in the aqueous phase was 5–100 times higher than blank readings. Inter-assay variability was <10%, and intra-assay variability was 5%. The limit of quantitation was 100 pmol of 6-hydroxychlorzoxazone, which, in a standard incubation mixture containing 500 μM chlorzoxazone, corresponded to 0.02% metabolism of the substrate. This was comparable with the limit of quantitation of the HPLC assay; hence, the extraction procedure and HPLC method for measuring chlorzoxazone 6-hydroxylation were comparable in terms of their sensitivity.

**Fig. 3.** Effect of concentration on the aqueous solubility of 6-[14C]hydroxychlorzoxazone following two consecutive extractions with dichloromethane.

6-[14C]Hydroxychlorzoxazone (250–2000 μM, specific activity 61 mCi/mol) was added to 1-ml mock incubation mixtures as described in Materials and Methods. After adjusting the pH to 8.5, the aqueous phase was extracted twice with 6 ml of dichloromethane. The amount of 6-[14C]hydroxychlorzoxazone remaining in the aqueous phase was determined by liquid scintillation spectrometry.

**Fig. 4.** Effect of incubation time and protein concentration on the 6-hydroxylation of [14C]chlorzoxazone by human liver microsomes.

Human liver microsomes pooled from seven individuals were incubated with [14C]chlorzoxazone (500 μM, 0.5 μCi/incubation) for different times (5–80 min with 0.4 mg of protein/ml) or at different protein concentrations (0.1–1.6 mg/ml for 20 min). Formation of 6-[14C]hydroxychlorzoxazone was determined by the solvent extraction procedure, as described in Materials and Methods.

**Fig. 5.** Sample-to-sample variation in [14C]chlorzoxazone 6-hydroxylation by human liver microsomes: comparison of the solvent extraction and HPLC procedures.

Human liver microsomes (0.4 mg) from eight donors and a pooled sample prepared from seven individuals were incubated with [14C]chlorzoxazone (500 μM, 0.5 μCi/incubation) for 20 min. Rates of formation of 6-[14C]hydroxychlorzoxazone were determined by the solvent extraction and HPLC procedures, as described in Materials and Methods. Data from the pooled sample were not included in the correlation analysis (shown in the inset).

**[14C]Chlorzoxazone 6-Hydroxylation by Human Liver Microsomes.** Eight samples of human liver microsomes and a pooled sample were incubated with [14C]chlorzoxazone, and the rates of 6-hydroxylation were determined by both the solvent extraction and HPLC procedure. As shown in fig. 5, the rates and sample-to-sample variation in the 6-hydroxylation of chlorzoxazone determined by the solvent extraction were the same as those determined by the HPLC procedure (r = 0.99). In addition, the sample-to-sample variation in 4-nitrophenol hydroxylase activity (30 μM 4-nitrophenol) was also highly correlated (r = 0.95) with the sample-to-sample variation in the 6-hydroxylation of chlorzoxazone in these microsomal samples (data not shown).

**Kinetics of [14C]Chlorzoxazone 6-Hydroxylation.** A pool of human liver microsomes (from seven individuals) was incubated with various concentrations of [14C]chlorzoxazone (10–80 μM), and the initial rates of 6-hydroxylation were determined by both the solvent extraction and HPLC procedures. As shown in fig. 6, the pool of human liver microsomes catalyzed the 6-hydroxylation of [14C]chlorzoxazone with an apparent \( K_m \) of \( 30 \text{ μM} \) and \( V_{max} \) of 3600 pmol/mg protein/min, regardless of the analytical procedure. These data are in agreement with those of Peter et al. (1990) who reported \( K_m \) values of 39 ± 7 μM and \( V_{max} \) values ranging from 1100 to 5900 pmol/mg protein/min, respectively, for chlorzoxazone 6-hydroxylation by human liver microsomes.

**Inhibition of [14C]Chlorzoxazone 6-Hydroxylation.** One application of the solvent extraction method is to facilitate the screening of drugs and new chemical entities as inhibitors of CYP2E1. Therefore, the utility of this method for screening CYP2E1 inhibitors was examined with 4-methylpyrazole, a known inhibitor of CYP2E1 (Feierman and Cederbaum, 1987; Halpert et al., 1994). As shown in fig. 7, 4-methylpyrazole inhibited the 6-hydroxylation of [14C]chlorzoxazone noncompetitively with a \( K_i \) value of 2.5 μM, regardless of
whether 6-hydroxychlorzoxazone was quantified by the solvent extraction or HPLC procedure.

**[14 C]Chlorzoxazone 6-Hydroxylation by cDNA-Expressed P450 Enzymes.** The extraction procedure was used to examine the rate of [14 C]chlorzoxazone 6-hydroxylation by a panel of human cDNA-expressed P450 enzymes, and the results are shown in fig. 8. Of the P450 enzymes examined, CYP2E1 catalyzed the highest rate of [14 C]chlorzoxazone 6-hydroxylation, followed by CYP1A1. Although CYP1A2 was a relatively poor catalyst of chlorzoxazone 6-hydroxylation, this enzyme has nevertheless been implicated in the 6-hydroxylation of chlorzoxazone by human liver microsomes, particularly at low substrate concentrations (Ono et al., 1995). Therefore, experiments were conducted to evaluate the contribution of CYP1A enzymes to the 6-hydroxylation of chlorzoxazone by human liver microsomes.

**Chlorzoxazone 6-Hydroxylation: CYP2E1 vs. CYP1A.** To evaluate the contribution of CYP1A1 and/or CYP1A2 to the 6-hydroxylation of chlorzoxazone by human liver microsomes, several water-soluble inhibitors were evaluated for their ability to differentially inhibit CYP1A and CYP2E1 (Draper et al., 1996b) (only water-soluble inhibitors were evaluated to avoid the use of organic solvents, most of which tend to inhibit CYP2E1). As shown in fig. 9, papaverine competitively inhibited the O-dealkylation of 7-ethoxyresorufin (a marker of CYP1A activity) with a $K_i$ value of 40 $\mu$M, but it did not competitively inhibit the 6-hydroxylation of chlorzoxazone by human liver microsomes ($K_i > 4,000$ $\mu$M). In this experiment, the concentration of each substrate corresponded to $\frac{1}{4}K_m$, $K_m$, and $4 \times K_m$, which corresponded to 0.125, 0.5, and 2 $\mu$M in the case of 7-ethoxyresorufin and 7.5, 30, and 120 $\mu$M in the case of chlorzoxazone.

Weresas CYP2E1 levels are relatively constant from one sample of human liver microsomes to the next (they vary ~3-fold, as shown in fig. 5), the levels of CYP1A2 vary more than an order of magnitude (Pearce et al., 1996). The effects of papaverine on chlorzoxazone 6-hydroxylation and 7-ethoxyresorufin O-dealkylation were evaluated with four samples of human liver microsomes (three individual samples and a pooled sample). The three individual samples (nos. 15, 19, 21) were selected because they all contain comparatively low and constant levels of CYP2E1 and yet contain low, medium, and high levels of CYP1A2. For this experiment, the concentration of 7-ethoxyresorufin was 5 $\mu$M (10 times the $K_m$ value), whereas the concentration of chlorzoxazone was 3 $\mu$M (5 times the $K_m$ value). Under these conditions, papaverine (400 $\mu$M) inhibited the O-dealkylation of 7-ethoxyresorufin by 50% and inhibited the 6-hydroxylation of chlorzoxazone by 15%, as shown in fig. 10. The significance of these results is discussed later. However, it should be noted that if 400 $\mu$M papaverine inhibited the O-dealkylation of 7-ethoxyresorufin by 50%...
Pooled human liver microsomes (0.4 mg) from seven individuals were incubated for 20 min with [14C]chlorzoxazone (7.5–120 μM, 0.5 μCi/incubation) in absence and presence of papaverine (200, 400, or 600 μM). Rates of formation of 6-[14C]hydroxychlorzoxazone were determined by the solvent extraction procedure, as described in Materials and Methods. Additionally, pooled human liver microsomes (0.4 mg) from seven individuals were incubated for 2 min with 7-ethoxyresorufin (0.125–2 μM) in absence and presence of papaverine (100, 200, or 400 μM). Rates of formation of resorufin were determined by fluorescence spectroscopy.

The sample-to-sample variation in chlorzoxazone 6-hydroxylation was examined at three substrate concentrations (namely 1/10 Km, Km, and 10 × Km, which corresponded to 3, 30, and 300 μM). As shown in fig. 11, the sample-to-sample variation in the rate of chlorzoxazone 6-hydroxylation at 300 μM was highly correlated with the rates at 3 and 30 μM chlorzoxazone (r = 0.99 and 0.95, respectively).

[14C]Chlorzoxazone 6-Hydroxylation by Rat Liver Microsomes. Liver microsomes from rats treated with prototypical P450 enzyme inducers were incubated with [14C]chlorzoxazone, and the rates of 6-hydroxylation were determined by both the solvent extraction and HPLC procedure. As shown in fig. 12, the CYP2E1 inducers, isoniazid and streptozotocin, and the CYP1A inducers, β-naphthoflavone, 3-methylcholanthrene, and Aroclor 1254, caused a 2–3-fold increase in chlorzoxazone 6-hydroxylase activity regardless of the analytical method. The rates of chlorzoxazone 6-hydroxylation determined by the solvent extraction and HPLC procedures correlated well with each other (r = 0.96).

Discussion

Chlorzoxazone 6-hydroxylation activity is routinely used to estimate the levels of CYP2E1 in human liver microsomes and for screening drugs and other chemicals as potential inhibitors of this enzyme. The aim of this study was to develop a solvent extraction (non-HPLC) procedure for measuring chlorzoxazone 6-hydroxylation activity. This procedure takes advantage of the fact that chlorzoxazone is metabolized by human and rat liver microsomes to a single product, namely 6-hydroxylchlorzoxazone. Incubations were carried out with [14C]chlorzoxazone, which is commercially available. The metabolite, 6-[14C]hydroxylchlorzoxazone, was separated from unreacted [14C]chlorzoxazone by adjusting the pH of the aqueous medium to 8.5 and extracting the parent compound with dichloromethane (2 × 6 ml). The extraction of unreacted [14C]chlorzoxazone was virtually quantitative (>99.9%); consequently, the solvent extraction procedure provides a very sensitive means of measuring chlorzoxazone 6-hy-
with the CYP2E1 inducers isoniazid and streptozotocin. Because human liver microsomes contain little or no CYP1A1 (Murray et al., 1993; Schweikl et al., 1993), this enzyme is thought to contribute negligibly, if at all, to the 6-hydroxylation of chlorzoxazone by human liver microsomes, as previously reported by Yamazaki et al. (1995). Furthermore, Carriere et al. (1993) reported that, at a substrate concentration of 400 μM, the turnover number for chlorzoxazone 6-hydroxylation by CYP2E1 is 10 times greater than that for CYP1A1 (25 vs. 2.5 min⁻¹). These results are in good agreement with those shown in fig. 8, where various cDNA-expressed P450 enzymes were incubated with 500 μM chlorzoxazone.

Ono et al. (1995) reported recently that human recombinant CYP1A2 catalyzes the 6-hydroxylation of chlorzoxazone at one-tenth the rate catalyzed by CYP2E1 (1.7 vs. 17.5 min⁻¹). These results contrast with those reported by Yamazaki et al. (1995) and Carriere et al. (1993), who showed that CYP1A2 has virtually no capacity to catalyze the 6-hydroxylation of chlorzoxazone (turnover number 0.02 min⁻¹ or less). The results of this study are consistent with those reported by Yamazaki et al. (1995) and Carriere et al. (1993). As shown in fig. 8, CYP2E1, CYP1A1, and CYP1A2 catalyzed the 6-hydroxylation of chlorzoxazone with turnover numbers of 5.98, 1.95, and 0.33, respectively. Interestingly, all of the cDNA-expressed P450 enzymes examined seemed to catalyze low rates of [¹⁴C]chlorzoxazone 6-hydroxylation. This may be an artifact of the B-lymphoblast expression system because even microsomes from control cells (i.e. B-lymphoblastoid cells that had not been transfected with recombinant vector) catalyzed the 6-hydroxylation of [¹⁴C]chlorzoxazone. According to the manufacturer (Gentest), the microsomes from B-lymphoblastoid cells may contain low levels of CYP1A1, which may be the source of the low chlorzoxazone 6-hydroxylase activity in these microsomal preparations.

Ono et al. (1995) suggested that CYP1A2 contributes to the 6-hydroxylation of chlorzoxazone by human liver microsomes, particularly at low substrate concentrations. The results of this study do not support this view. First, under conditions that would be expected to completely inhibit CYP1A2, papaverine (400 μM) inhibited the 6-hydroxylation of chlorzoxazone by only 15%, even at a substrate concentration of 3 μM (i.e. v/Vo Km). However, the degree of inhibition was independent of the levels of CYP1A2 in human liver microsomes. In other words, papaverine inhibited the 6-hydroxylation of chlorzoxazone by 15% even in liver microsomes that contained negligible levels of CYP1A2, which suggests that the 15% inhibition observed represents weak inhibition of CYP2E1. Furthermore, the sample-to-sample variation in chlorzoxazone 6-hydroxylase activity was not influenced by substrate concentration (over the range of 3 to 300 μM) and was highly correlated with the hydroxylation of 4-nitrophenol, all of which is consistent with a single enzyme, namely CYP2E1, being largely responsible for the 6-hydroxylation of chlorzoxazone by human liver microsomes.

The solvent extraction procedure was developed in part to facilitate the rapid screening of drugs and new chemical entities as inhibitors of CYP2E1. The solvent extraction procedure is considerably less time consuming than the conventional HPLC procedure, yet it is comparable in terms of sensitivity and reproducibility. The solvent extraction procedure avoids the occasional problem of test articles co-eluting with 6-hydroxychlorzoxazone and thereby interfering with its quantitation by HPLC. In conclusion, the solvent extraction procedure for measuring the 6-hydroxylation of [¹⁴C]chlorzoxazone should be useful for measuring CYP2E1 levels in human liver microsomes and for identifying drugs and other chemicals that inhibit this enzyme.
References


