Short Communication

Ethosuximide is Primarily Metabolized by CYP3A when Incubated with Isolated Rat Liver Microsomes

ABSTRACT:

The cytochrome P450 (CYP) subfamily responsible for ethosuximide metabolism was investigated by HPLC assay of ethosuximide incubations with isolated rat liver microsomes from control rats and from rats treated with inducing agents to enrich hepatic microsomes in selected CYP isoforms. Inducing agents included β-naphthoflavone (BNF, CYP1A inducer), phenobarbital (PB, CYP2B/2C/3A), isoniazid (INH, CYP2E1), clotrimazole (CTZ, CYP3A), clofibrate (CLO, CYP4A), and an imidazole CTZ-analog known as CDD3543 (CYP3A). Incubations with BNF, INH, CTZ, and control microsomes showed significantly (p<0.05) more metabolite produced by CTZ microsomes vs. BNF, INH, and control microsomes at 10, 30, 60, and 120 min incubation. Ethosuximide metabolite levels generated by CTZ microsomes at 120 min were 36.5 times those of control microsomes. Correspondingly, ethosuximide concentrations were significantly (p<0.05) lower for incubations with the CTZ microsomes compared with BNF, INH, and control microsomes at 60 and 120 min. Sixty-minute incubations with all microsome groups exhibited significantly (p<0.05) higher metabolite formation rates (nmol/nmol CYP/min) for CTZ (11.8x control) and PB (9.6x control) microsomes vs. all other groups. Antibody inhibition experiments demonstrated ethosuximide metabolite levels for PB microsomes were not affected by CYP2B1 antibodies, whereas CYP3A2 antibodies reduced metabolite levels for both PB and CTZ microsomes by over 80%. These results indicate CYP3A is primarily responsible for ethosuximide metabolism in rats.

Cytochrome P450 (CYP) enzymes play a key role in the metabolism of many exogenous and endogenous substances (Porter and Coon, 1991), with CYP3A being one of the most important CYP subfamilies for drug metabolism (Wright and Paine, 1994). Several drugs have been investigated as probes of CYP3A activity, including midazolam (Thummel et al., 1994), erythromycin (Lown et al., 1992), nifedipine, dapsone, lidocaine (Watkins, 1994), and quinidine (Guengerich et al., 1986). Cortisol 6β-hydroxylation has also been employed as a CYP3A expression marker (Horsmans et al., 1992).

Ethosuximide is an anticonvulsant that has been investigated as an alternative probe for CYP3A activity (Bachmann et al., 1992; Bachmann and Jauregui, 1993). Ethosuximide is well suited for use as a probe molecule in that it can be given orally, its clearance can be estimated from a single plasma or saliva sample (Bachmann and Jauregui, 1993), and it is not significantly bound by plasma proteins. In vivo studies have indicated that ethosuximide is principally oxidized by CYP3A in rats (Bachmann et al., 1992) and humans (Bachmann and Jauregui, 1993). The primary ethosuximide metabolite has been shown to have an α-hydroxy group on the ethyl side chain, 2-(1-hydroxyethyl)-2-methylsuccinimide (Horning et al., 1973; Peterson, 1980; Maurer, 1990; Millership et al., 1993; Millership et al., 1995; Pisani et al., 1995), accounting for 40–60% of the administered drug excreted into the urine in rats and humans (Horning et al., 1973; Millership et al., 1993; Pisani et al., 1995). Other forms of the drug excreted in urine include unchanged drug (about 10% of administered drug), glucuronide conjugates (20–40%), a ring-hydroxylated form (2-ethyl-3-hydroxy-2-methylsuccinimide, near 7%), a form with a β-hydroxy group on the ethyl side chain (2–3%), and dihydroxy and carboxylic acid forms detected at lower levels (Horning et al., 1973; Pisani et al., 1995).

The present study was undertaken to determine how in vitro ethosuximide biotransformation and ethosuximide metabolite formation are affected when ethosuximide is incubated with isolated rat microsomes enriched with different CYP subfamily enzymes. An HPLC method developed in our laboratory was used to measure the levels of ethosuximide and its primary metabolite after in vitro incubation of ethosuximide with isolated rat liver microsomes enriched in selected CYP isoforms. Incubation experiments included time series measurements with a selected subset of the isoform-enriched microsomes. 60-min incubations with each type of isoform-enriched microsome considered in this study, and inhibition measurements made with specific CYP subfamily antibodies.

Materials and Methods

Chemicals. NADP and 3,3-dimethylglutaric acid were purchased from Aldrich Chemical Company (Milwaukee, WI); methyl cellulose 1500 USP from RUGER Chemical Company (New York, NY); rabbit anti-rat CYP2B and CYP3A2 antisera from Gentest Corporation (Woburn, MA); Bio-Rad protein assay reagent from Bio-Rad Laboratories (Hercules, CA); and HPLC grade acetonitrile from Fisher Scientific (Pittsburgh, PA); all other chemicals were the highest grade available from Sigma Chemical Company (St. Louis, MO). HPLC grade water was generated on a Millipore MilliQ system (Bedford, MA).

Preparation of Liver Microsomes. Fifty male Sprague-Dawley 230–270 g rats from Harlan Sprague-Dawley (Indianapolis, IN) were divided randomly into seven groups. Inducing agents suspended in 1% methyl cellulose (MC) were administered by single daily gavage for 3 days. Seven control animals received only MC suspension, eight animals received BNF (a CYP1A inducer), eight received PB (a CYP2B/2C/3A inducer), seven received CTZ (a CYP3A inducer), six received INH (a CYP2E1 inducer), seven received CLO, two received PB (a CYP2B/2C/3A inducer), seven received CTZ (a CYP3A inducer), six received INH (a CYP2E1 inducer), seven received CLO,
a CYP4A inducer), and seven received CDD3543, a CTZ-analog and CYP3A inducer). Doses of 100 mg/kg/day were used for BNF, CTZ, and INH, while 50 mg/kg/day was used for PB, CLO, and CDD3543. Livers were excised 24 hr after the last dose except in imidazole-treated animals (CTZ and CDD3543) which were excised 48 hr after the last dose (Ritter and Franklin, 1987). Animals were sacrificed under CO2 anesthesia after an overnight fast. The liver was quickly removed, perfused with 1.15 g/ml potassium chloride solution, homogenized in four volumes of ice-cold 1.15 g/ml potassium chloride solution, centrifuged at 10,000 × g for 20 min at 4°C, and the supernatent was then further ultracentrifuged at 105,000 × g for 60 min at 4°C. The microsomal pellets were resuspended in 10 ml of 100 mM pH 7.4 potassium phosphate buffer containing 0.1 mM EDTA. Protein and CYP levels were measured by standard methods (Bradford, 1976; Omura and Sato, 1964). There were no significant differences in initial or final animal weights or final liver weights between any of the animal treatment groups. CYP levels per mg protein were significantly higher for all treated animal groups vs. controls, with nmol CYP/mg protein levels of 0.40 ± 0.22, 1.38 ± 0.28, 1.95 ± 0.22, 1.00 ± 0.22, 1.15 ± 0.17, 2.29 ± 0.51, and 3.54 ± 0.68, respectively, for microsomes from control, PB, INH, CLO, CTZ, and CDD3453 treated animals. Microsomal suspensions were stored at −80°C until use.

Incubation Time Series. Incubations with microsomes from control, BNF, INH, CTZ, and CLO treated animals were carried out aerobically at 37°C in glass culture tubes. NADPH generating system was prepared by adding 5.4 mg NADP, 27.2 mg glucose-6-phosphate, and 1.0 unit glucose-6-phosphate dehydrogenase to 1.0 ml of 60 mM magnesium chloride and 0.1 mM EDTA at 37°C. Microsome suspensions were thawed and diluted with 100 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA to a final concentration of 10 mg protein/ml. Seven hundred microliters of 1.0 M 7.4 pH potassium phosphate buffer, 100 µl of 10 mM aqueous ethosuximide solution, 100 µl of 10 mg protein/ml microsomal suspension, and 100 µl of NADPH generating system solution were then added to each culture tube and incubated at 37°C with shaking for 0, 10, 30, 60, or 120 min. Metabolism was stopped at the end of the incubation period by placing the tube on ice and adding 50 µl of ice-cold 0.2 g/ml trichloroacetic acid (TCA) solution. Five hundred microliters of an aqueous 200 µg/ml 3,3-dimethylglutarimide solution was then added to each tube as an HPLC internal standard. Each tube was centrifuged at 9600 × g for 10 min, and the supernatent was filtered through a 0.2 µm nylon syringe filter. Samples were found to be stable for at least one month when stored at 4°C. Three tubes were prepared at each time period for each inducing agent using microsomes from a single animal source for each agent, with duplicate HPLC assays performed on 0 and 60 min samples and single sample assays for other incubation time samples.

60-Minute Incubations. Sixty-minute incubations were carried out with all microsome groups using the procedures described for the time series incubations. Microsomes from three different animals were used for each treatment group, with three incubations performed for each animal source, yielding nine samples per treatment group with a single HPLC assay performed on each sample.

Antibody Inhibition. Microsomes from CTZ treated animals were incubated after exposure to anti-CYP3A2 antibodies, whereas microsomes from PB treated animals were tested after exposure to either anti-CYP2B1 or anti-CYP3A2 antibodies. Microsomal suspensions were diluted to 2 mg protein/ml with 100 mM phosphate buffer containing 0.1 mM EDTA to match supplier suggested antibody conditions. Fifty microliters of this microsomal suspension were then preincubated at room temperature for 30 min with either 0, 10, 20, or 50 µl of anti-CYP rabbit antiserum. An appropriate volume of normal rabbit serum was added to keep the total rabbit serum volume in each tube at 50 µl. After preincubination, 340 µl of 0.1 M pH 7.4 phosphate buffer, 10 µl of 10 mM ethosuximide solution, and 50 µl of NADPH generating solution was added to each tube and incubated at 37°C for 120 min. After incubation, tubes were placed on ice, 250 µl of ice-cold 0.2 g/ml TCA solution was added, 250 µl of HPLC internal standard solution was added, and samples were prepared as described previously. Triplicate HPLC assays were performed on all samples.

HPLC Assay Procedure. Samples were assayed by reversed phase HPLC using a 20-µl injection onto a Waters HPLC system containing two Model 501 pumps, a Model U6K injector with 2.0 ml sample loop, a Model 484 UV detector set at 195 nm detection, and a NovaPak 3.9 mm × 15 cm C18 column. Mobile phase consisted of water as phase A and acetonitrile as phase B flowing at a constant flow rate of 1 ml/min. The initial mobile phase composition was 75% phase A and 25% phase B, and the mobile phase was isocratically eluted for 10 min, followed by a linear gradient to 80% phase B over 30 min. The detector was set at 195 nm, and a series of HPLC chromatograms for each incubation time was recorded. A representative chromatogram for a 120 min CTZ microsome incubation sample is shown in Fig. 1. The HPLC chromatograph is a useful tool for the analysis of microsome incubation samples because it allows for the rapid identification of metabolites and provides information on the relative concentrations of different metabolites. The chromatogram in Fig. 1 shows the relative absorbance at 195 nm vs. the elution time after sample injection. Peaks are: M, presumed minor metabolite; p, primary metabolite; I, internal standard, ethosuximide. Plot A is a chromatograph for a 120 min CTZ microsome incubation sample. Plot B shows a series of chromatograms for CTZ samples incubated for different periods of time. Some peaks in Plots B have been graphically truncated for clarity.

**Fig. 1.** HPLC chromatograms of microsome incubation samples.
at a rate of 2.0 ml/min, with a gradient elution profile of 3.0–35.0%B linear ramp from 0–6.4 min, 35.0–3.0%B linear ramp from 6.4–7.0 min, followed by a 9-min washout period. Standards were prepared on ice with the same reagents as the incubation samples, but the TCA solution was added first and the ethosuximide solution was adjusted to give equivalent incubation media ethosuximide concentrations of 2, 20, 200, 1000, and 2000 nmol/ml. Control samples at 2.0, 200, and 2000 nmol/ml were prepared similarly by separate weighing. A two-parameter quadratic equation with zero intercept was used to produce a calibration curve for the ethosuximide to internal standard peak area ratio. As there was no source of the primary ethosuximide metabolite, the metabolite was assumed to have the same peak area response per mole as ethosuximide and the ethosuximide calibration curve was used to quantify the metabolite level. Relative error and within/between-day coefficients of variation were determined by multiple measurements on control samples and on samples produced by incubation with CTZ microsomes for 0, 30, and 120 min. To identify the ethosuximide metabolite structure, Electrospray Ionization-Liquid Chromatography-Mass Spectrometry (ESI-LCMS) analysis was performed on a 240 min CTZ microsome incubation sample using a similar gradient elution profile with 0.1% ammonium hydroxide post-column ionization.

Statistical Analysis. Results are expressed as mean ± SD. Differences between groups were assessed by one-way ANOVA with Tukey's post-hoc analysis using \( p < 0.05 \) as the criterion for statistical significance. Linear regression was employed to evaluate correlations between continuous variables.

Results and Discussion

HPLC Assay Method Validation. Because it is difficult to detect the small changes in ethosuximide levels that occur during in vitro microsomal incubations, it became necessary to measure the corresponding increase in ethosuximide metabolite levels. To this end, a novel HPLC method was developed to quantify the levels of ethosuximide and its primary metabolite in incubation samples. This is the first reported HPLC method for analysis of an ethosuximide metabolite, as all previously published ethosuximide metabolite measurements have been made using GC-MS (Horning et al., 1973; Pettersen, 1980; Millership et al., 1993; Millership et al., 1995). An example chromatogram illustrating clear separation of ethosuximide, primary metabolite, and internal standard peaks is provided in fig. 1A. A smaller peak apparently resulting from a less prominent ethosuximide metabolite appears near 4.5 min elution but was too small to consistently quantify. Fig. 1B illustrates the metabolite peak increases in size as the period of CTZ microsome incubation increases. Ethosuximide measurement accuracy ranged from \(-5.2\%\) to \(+3.7\%\) over the entire 2.0–2000 nmol/ml range, while within- and between-day variation over these concentrations ranged from \(0.6–6.3\%\). Primary metabolite within- and between-day variation ranged from \(0.7\%–6.3\%\) over the measured concentration range of 3.5–153 nmol/ml, with the exception of a 13.7% within-day variation at the lowest measured concentration.

ESI-LCMS yielded an intact ethosuximide \([M-H]^-\) ion at \(m/z\) 140 corresponding to a proton loss, with a decomposition fragment ion at \(m/z\) 112 produced by loss of ethylene via the ethyl side chain. The primary metabolite yielded an intact ion at \(m/z\) 156, supporting the presumption that it is a monohydroxyated form of ethosuximide. The metabolite fragment ion appeared at \(m/z\) 112, which can best be explained by loss of acetaldehyde via a hydroxylated ethyl side chain. Presence of the hydroxyl group anywhere other than the ethyl side chain would cause the loss of ethylene from the ethyl side chain, which would yield a metabolite fragment ion at \(m/z\) 128 that was not detected. These results then indicate the metabolite peak is caused by a monohydroxylated metabolite of ethosuximide with a hydroxy group on the ethyl side chain. The ESI-LCMS analysis could not differentiate between \(\alpha\) - and \(\beta\)-hydroxylated metabolite forms, but previous GC-MS results (Horning et al., 1973; Millership et al., 1993; Pisani et al., 1995) indicate the \(\alpha\)-hydroxyl form should be 10–20 times more predominant and hence should be the main source of the metabolite peak even if the \(\alpha\)- and \(\beta\)-hydroxylated forms co-elute.

Hence the HPLC method was shown to provide consistent quantitation of ethosuximide and its primary metabolite, although the absolute quantitation of the metabolite measurements could not be confirmed because a metabolite standard was not available. Because this study was concerned with relative comparisons of metabolite levels between samples, however, absolute quantitation was not necessary.

Microsome Incubations. Fig. 2A shows only CTZ microsomes produce detectable drops in ethosuximide levels in the time series experiments, with ethosuximide concentrations for CTZ microsomes significantly lower at 60 and 120 min compared with those for each of the other microsome treatment groups. Linear regression analysis of the ethosuximide concentration vs. time for the 0–60 min samples of each microsome group shows only CTZ microsome values correlate strongly with incubation time \((r = 0.9965)\). Other microsome groups
exhibit weak correlations and yield essentially horizontal fitted lines. Fig. 2B shows the CTZ microsomes produce significantly higher metabolite levels than all other microsome treatment groups at each incubation time period. At 120 min incubation, the metabolite level for CTZ microsomes was 36.5-fold that of control microsomes. Metabolite levels for all microsomes tested correlated strongly with incubation time over the first 60 min.

Fig. 3A shows 60-min incubations with CTZ, CDD3543, and PB microsomes yielded significantly lower ethosuximide levels than all other treatments. Corresponding metabolite formation rates on a nmol/nmol P450/min basis in Fig. 3B were elevated for CTZ (11.8x control), PB (9.6x control), and CDD3543 (5.4x control) microsomes, with each significantly different than controls.

Fig. 4 shows the metabolite formation inhibition for each antibody/microsome combination studied. Little or no inhibition occurred for PB microsomes with anti-CYP2B1 antibodies, while both PB and CTZ microsomes exhibited systematic decreases in metabolite formation with anti-CYP3A2 antisera volume. Both PB and CTZ microsomes exhibited more than 80% inhibition with 50 µl of anti-CYP3A2 antisera. Hence time series and 60-min incubation experiments demonstrated that microsomes enriched in CYP3A (PB, CTZ, and CDD3543 treatment) had consistently higher rates of ethosuximide disappearance and metabolite formation than control microsomes or CYP1A (BNF treatment), CYP2E1 (INH treatment), or CYP4A (CLO treatment) enriched microsomes. As PB induces CYP2B as well as CYP3A, immunoinhibition studies were performed and showed anti-CYP2B1 antibodies had no effect on metabolite formation with PB microsomes, whereas anti-CYP3A2 antibodies inhibited metabolite formation for both PB and CTZ microsomes. These results indicate that CYP3A is primarily responsible for the ethosuximide biotransformation to hydroxyethyl-ethosuximide in isolated rat microsomes, corroborating an earlier in vivo study (Bachmann et al., 1992) that suggested CYP3A plays a pre-eminent role in the metabolism of ethosuximide in rats. Similar in vitro studies are underway with human microsomes.

Acknowledgments. The authors wish to thank Dr. Joe Zirrolli of Hauser Laboratories for the ESI-LCMS analysis, Ning Peng and Allison Glinka for their HPLC assistance, and Dr. Paul Erhardt and Zhiyong Hu of the Center for Drug Design and Development (CD3) for their work on the synthesis of ethosuximide metabolites.

Department of Pharmacology, College of Pharmacy, The University of Toledo

JEFFREY G. SARVER
KENNETH A. BACHMANN
DALING ZHU2
WIESLAW A. KLIS3

2 Current address: Cardiovascular Research Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226.
3 Current address: Department of Microbiology and Immunology, Medical College of Ohio, 3000 Arlington Ave., Toledo, OH 43614.
References


