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Inhibition of microsomal phenobarbital metabolism by valproic acid

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Valproic acid (di-*n*-propylacetic acid, 2-propylpentanoic acid, 2-propylvaleric acid) is an antiepileptic drug approved for use in the United States by the U.S. Food and Administration in 1978. A branched, short-chain fatty acid, it differs from the other antiepileptic agents in not being a heterocyclic compound. Its pharmacological and therapeutic properties have been reviewed recently [1–3]. Valproate is generally used in conjunction with other antiepileptic drugs, and it has been implicated in several drug–drug interactions [2, 4–6]. One of these interactions involves valproate-induced elevation of plasma phenobarbital levels [2, 4–6], which can lead to various degrees of undesirable sedation. The mechanism for this interaction has not been defined. In our study of four epileptic patients [7, 8], which will be described more fully,* the addition of valproate to the existing phenobarbital regimen resulted in a 30–70 per cent increase in plasma phenobarbital levels. Increases in plasma phenobarbital levels were paralleled by increases in phenobarbital elimination half-lives, whereas decreases in plasma phenobarbital clearances paralleled decreases in elimination rate constants for phenobarbital. These data suggest that the mechanism behind the valproate-caused elevation of plasma phenobarbital levels is an inhibition of phenobarbital metabolism. Therefore, in this study we examined in more detail the nature of valproate inhibition of phenobarbital metabolism, using an *in vitro* hepatic microsomal system from phenobarbital-treated rats.

Materials. Sodium phenobarbital was obtained from Merck & Co. (Rahway, NJ) and *p*-hydroxyphenobarbital from the Aldrich Chemical Co. (Milwaukee, WI). Sodium valproate was supplied by Dr. A. O. Geiszler (Abbott Laboratories, North Chicago, IL), 5,5-diphenylbarbituric acid by Dr. A. Raines (Department of Pharmacology, Georgetown University School of Medicine and Dentistry, Washington, DC), and *m*-hydroxyphenobarbital by Dr. K. H. Dudley (Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC). NADPH was purchased from Boehringer–Mannheim Biochemicals (Indianapolis, IN). All organic solvents were from Burdick and Jackson Laboratories, Inc. (Muskegon, MI), and all other chemicals were of reagent grade.

Animals. Adult male Holtzman rats weighing about 200 g each were obtained from Charles River Breeding Laboratories (Wilmington, MA). For 7 days prior to use, animals were kept in a separate animal room where exposure to pharmacologically active compounds such as insecticides and solvents was prevented. They had free access to food and water. To simulate chronic phenobarbital treatment in humans, the rats were treated with an aqueous solution of sodium phenobarbital (75 mg/kg) administered intraperitoneally every day for 3 days, the last dose being administered 24 hr before the animals were killed. On day 4, they were killed and hepatic microsomes were isolated as described previously [9]. Cytochrome P-450 content was determined by ultraviolet-visible difference spectroscopy, according to the method of Omura and Sato [10].

Microsomal reactions. Since *p*-hydroxyphenobarbital is the major metabolite of phenobarbital in man, its rate of formation was studied. Conditions for microsomal reactions were optimized with respect to ionic strength, pH of the buffer, and NADPH concentration. Reactions were carried out under conditions in which the rates of *p*-hydroxyphenobarbital formation were linear with respect to reaction time (8 min) and enzyme concentration (1.83 nmoles of cytochrome P-450 or 0.702 mg of microsomal protein). Total reaction volume was 5.0 ml, and reactions were carried out at 37° in 0.1 M potassium phosphate buffer, pH 7.50, in the presence of 0.25 mM (final concentration) NADPH. After equilibration for 1 min at 37°, reactions were initiated by the addition of NADPH.

Assay. Details of the analytical procedure for quantitation of *p*-hydroxyphenobarbital generated in the microsomal reaction will be described in detail elsewhere.† Briefly, the microsomal reaction was terminated by the addition of cold benzene. A double benzene wash of the reaction mixture was used to eliminate some of the large substrate excess, which would interfere with the chro-

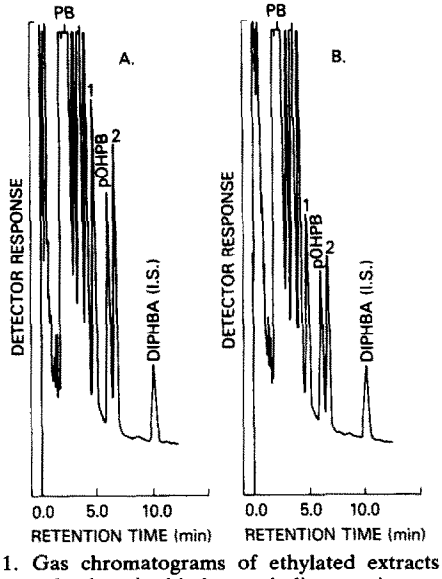

Fig. 1. Gas chromatograms of ethylated extracts from microsomal phenobarbital metabolic reactions carried out in the absence (A) and the presence (B) of valproate. The phenobarbital concentration in both reactions was 4.0 mM and the valproate concentration was 2.0 mM. A nitrogen selective detector was used. Peaks represent the residual phenobarbital (PB), the metabolite *p*-hydroxyphenobarbital (p-OHPB), and the internal standard (I.S.) diphenylbarbituric acid (DIPHBA). Peaks 1 and 2 represent two other metabolites of phenobarbital (see text). The amounts of *p*-hydroxyphenobarbital-formed·min^{-1}·(nmole P-450)$^{-1}$ in the reactions were 59.8 ng (A) and 43.9 ng (B).

* I. M. Kapetanović, H. J. Kupferberg, R. J. Porter, W. Theodore, E. Schulman and J. K. Penry, Mechanism of valproate-phenobarbital interaction in epilepsy, *Clin. Pharmacol. Ther.* (in press).

† I. M. Kapetanović and H. J. Kupferberg, Gas chromatographic and gas chromatographic–mass spectrometric determination of *p*-hydroxyphenobarbital extracted from plasma, urine and hepatic microsomes, *J. Pharm. Sci.* (in press).

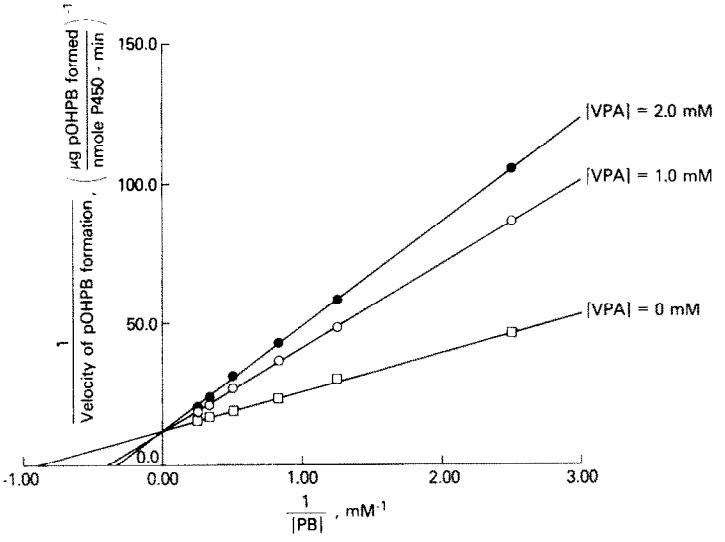

Fig. 2. Lineweaver–Burk plot of the inhibition of phenobarbital *p*-hydroxylation by valproate. Hepatic microsomes from phenobarbital-treated Holtzman rats were used. Typical data obtained at 0, 1.0 and 2.0 mM valproate are shown. Each point is the average value of a reaction carried out in duplicate.

matographic analysis. Then, diphenylbarbituric acid (internal standard) was added, and extraction was performed with ethyl acetate. The dried ethyl acetate extract was derivatized with ethyl iodide in acetonitrile in the presence of tetraethylammonium hydroxide. The derivatized extract was analyzed by gas chromatography, using a nitrogen selective detector. Chromatography was carried out isothermally at 210°, using a glass column (1.8 m × 2 mm, i.d.) packed with 2 per cent OV3 on 100/120 Supelcoport.

Gas chromatographic retention time of the peak ascribed to *p*-hydroxyphenobarbital was shown to be essentially the same as that of the reference *p*-hydroxyphenobarbital, even when the analyses were carried out using several column packings of different polarities. Spiking of the ethylated reaction extract with the ethylated reference *p*-hydroxyphenobarbital further substantiated the identical chromatographic characteristics of the two. The electron impact mass spectrum of the reference *p*-hydroxyphenobarbital showed prominent ions having *m/z* 332, 304, 303, 276, 275 and 190, with a relative abundance of 81, 63, 100, 20, 91 and 43 per cent respectively.* Furthermore, cross verification of the gas chromatographic quantitation method was performed by selected ion monitoring in the electron impact mode.* There was excellent correlation between the two methods.

Results and discussion. The velocity of *p*-hydroxylation of phenobarbital was followed as a function of phenobarbital concentration, and it was shown to follow Michaelis–Menten kinetics. Data were plotted as Lineweaver–Burk plots ($1/v$ vs $1/s$), and the kinetic parameters K_m and V_{max} were obtained from linear least squares regression lines. All microsomal reactions were performed in duplicate, and there was good correspondence between the duplicate reactions. Good correspondence was also evident for K_m and V_{max} values obtained on separate occasions. For example, K_m and V_{max} were, respectively, 1.16 ± 0.03 mM and 0.325 ± 0.008 nmole *p*-hydroxyphenobarbital formed · min^{-1} · $(nmole\ P\text{-}450)^{-1}$ (mean ± S.D. of three determinations). Virtually the same values for K_m and V_{max} were obtained from Woolf–Augistinsson–Hofstee plots (v vs v/s).

The addition of valproate to the microsomal reaction inhibited *p*-hydroxylation of phenobarbital. Gas chromatograms of extracts from the microsomal reaction carried out in the absence (Fig. 1A) and the presence (Fig. 1B) of valproate clearly illustrate this inhibition. The peak height of the metabolite, *p*-hydroxyphenobarbital, relative to the peak height of the internal standard, diphenylbarbituric acid, was markedly diminished when the reaction was carried out in the presence of valproate.

Two other metabolites of phenobarbital, in addition to *p*-hydroxyphenobarbital, were detected in the microsomal reaction extracts (Fig. 1A–B). Metabolite 1 was shown to correspond to *m*-hydroxyphenobarbital, on the basis of its having virtually the same chromatographic and mass spectrometric characteristics as the reference standard. Gas chromatographic retention time of metabolite 1 coincided with that of the reference *m*-hydroxyphenobarbital, even when separations were carried out using several column packings of different polarities. Spiking of the metabolite with the reference compound further substantiated the identical gas chromatographic characteristics of the two. The electron impact mass spectrum of the ethylated reference *m*-hydroxyphenobarbital showed prominent ions having *m/z* 332, 304, 276 and 190, with a relative abundance of 100, 98, 50 and 88 per cent, respectively. Selected ion monitoring showed essentially the same ratio of these ions for metabolite 1. Metabolite 2 may have been the ortho isomer, but its identity was not established. Valproate also inhibited production of these two metabolites (Fig. 1A–B).

The Lineweaver–Burk plot (Fig. 2) was used to characterize further the valproate-induced inhibition of phenobarbital *p*-hydroxylation. Increasing the concentration of valproate increased K_m, but it had no effect on V_{max}, which is typical of competitive inhibition. The inhibition constant, K_i, was calculated to be 1.20 mM.

We are not aware of any studies on microsomal metabolism of valproate. It is thought, however, to be metabolized *in vivo* in part by ω-oxidation [3, 11, 12], and microsomal cytochrome P-450 enzymes do carry out ω-oxidation of branched-chain fatty acids of various lengths [13–15]. Therefore, it is plausible that valproate be metabolized, in part, by microsomal systems, which could explain its competitive inhibition of phenobarbital metabolism.

* I. M. Kapetanović and H. J. Kupferberg, Gas chromatographic and gas chromatographic–mass spectrometric determination of *p*-hydroxyphenobarbital extracted from plasma, urine and hepatic microsomes, *J. Pharm. Sci.* (in press).

The therapeutic range of plasma phenobarbital and valproate levels is generally accepted as 10–30 μg/ml and 50–100 μg/ml respectively [16]. We have observed inhibition of phenobarbital metabolism by valproate in patients having phenobarbital levels of about 20 μg/ml and valproate levels of about 65 μg/ml. The K_m and K_i determined here for the rat hepatic microsomal system were, respectively, 1.16 mM (269 μg/ml) and 1.20 mM (173 μg/ml). Using plasma phenobarbital and valproate levels in patients for concentrations of substrate (s) and inhibitor (I), and using K_m, K_i and V_{max} values determined for the rat hepatic microsomal system, we calculated the $K_{m(app)}$ (the apparent K_m for p-hydroxylation of phenobarbital in the presence of valproate) by one form of the equation describing competitive inhibition,

$$K_{m(app)} = \frac{K_m}{K_i}(I) + K_m$$

Then using the Michaelis–Menten equation

$$v = \frac{V_{max}}{\frac{K_m}{(s)} + 1}$$

we calculated the velocity of the reaction (v) in the absence and presence of valproate by substituting the appropriate values for K_m and $K_{m(app)}$. A decrease of about 26 per cent in the velocity of phenobarbital p-hydroxylation would be expected under these conditions. In our study of patients [7, 8], we found 20–50 per cent decreases in plasma clearance and elimination rate constant for phenobarbital. Thus, by a gross extrapolation, there appears to be a reasonable correspondence between the findings from the *in vivo* study in humans and this *in vitro* study in rats. The inherent assumptions and limitations of such an extrapolation, however, are obvious, and it is used here only as a very crude comparison between two different systems.

In this study, Michaelis–Menten kinetics for phenobarbital p-hydroxylation were described in the absence and presence of valproic acid in an *in vitro* hepatic microsomal system from phenobarbital-treated rats. Valproic acid was shown to act as a competitive inhibitor in this reaction. These findings support and further characterize the mechanism that we have proposed for the valproate–phenobarbital interaction in epileptic patients as one of metabolic inhibition.

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