Glucuronidation Metabolic Kinetics of Valproate in Guinea Pigs: Nonlinear at Clinical Concentration Levels

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Purpose. Nonlinear conjugation metabolic rate of valproic acid (VPA) has been speculated previously from plasma elimination and liver concentration of VPA in guinea pigs. The purposes of the present study were to assess our speculation by direct measurement of VPA glucuronidation rate in vitro.

Methods. VPA at various concentrations (10–200 μg/ml) was incubated with guinea-pig liver homogenate, mitochondria or microsome in the presence of cofactor, uridine 5’-diphosphoglucuronic acid (UDPGA). The maximum glucuronidation rate (Vmax) and Michaelis-Menten constant (Km) of VPA were determined.

Results. On a body weight basis, the Vmax and the Km values of VPA glucuronidation estimated from liver homogenate were 1.8 μmol/min/kg and 0.3 μmol/ml, respectively; and that from microsome suspension were 1.2 μmol/min/kg and 0.16 μmol/ml, respectively. These data are comparable with the primary metabolic parameters observed from previous in vivo study. The glucuronidation clearance calculated from these parameters was 0.10–0.48 fraction of total clearance, which was in agreement with the reported data observed from clinical and animal urinary recoveries of VPA-G. The glucuronidation reaction was not detectable in mitochondria suspension.

Conclusions. The glucuronidation kinetics of VPA is nonlinear and saturable within clinical concentration range. Estimation of in vivo VPA glucuronidation kinetics from in vitro kinetic parameters is feasible.

KEY WORDS: valproate; metabolic kinetics; glucuronidation; Michaelis-Menten constant; nonlinear.

INTRODUCTION

Valproic acid (VPA) is now used worldwide as an anti-convulsant. Clinical studies have indicated that VPA may have a moderate inducing (1), an inhibitory effect (2) or cause no alterations (3–5) on its own metabolism. Studies in animals suggested that plasma concentration and duration of treatment may be important factors in determining the effects of VPA on its own metabolism (6,7). All of these suggestions were speculations from in vivo data (mostly glucuronide metabolite concentrations). The combined effects of concentration-dependent plasma protein binding and clearance (8), associated with the relative rate of oxidation and glucuronidation (9) of VPA should play an important role in the variability of reported metabolic kinetics. VPA is eliminated by hepatic metabolism predominantly through glucuronide conjugation and β-oxidation (9). Although the metabolic fate of VPA has been studied extensively in vivo, and, to a limited extent, in vitro, little is known about the conjugation metabolic kinetic of this drug. Granneman et al (9) proposed that glucuronidation and β-oxidation are primary pathways, whereas ω and γ-oxidation are secondary pathways. However, the suggestion has not been verified. This laboratory has estimated the metabolic characteristics of VPA from plasma concentration data in guinea pigs, where a biphasic pattern was demonstrated with the primary Km value being within therapeutic concentration range (8); and it was also found that the hepatic concentration of valproate glucuronide (VPA-G) was saturable (8). Evidence showed that the primary parameters were most likely to describe the conjugation metabolic rate, and the results could adequately interpret the inconsistent outcome of the effect of VPA on its own metabolic rate. These findings from in vivo study need to be verified by further study.

The purposes of the present study were to measure the glucuronide conjugation metabolic rate by direct reaction of the substrate, VPA, with liver-component (liver homogenate, mitochondria and microsome) in vitro, and to compare the result with that observed from previous in vivo study (8). The results may provide a fundamental basis for interpretation and prediction of the variable metabolic rate of VPA observed from clinical study.

MATERIALS AND METHODS

Chemicals

Sodium valproate crystalline powder was a gift from Ciba-Geigy Taiwan branch. Uridine 5’-diphosphoglucuronic acid (UDPGA) trisodium salt was purchased from Sigma (St. Louis, Mo. USA). Tris(hydroxymethyl)aminomethane (Tris) and other reagents were purchased from E. Merck (Germany).

Preparation of Liver Homogenate, Mitochondria, and Microsome

Guinea pigs (Experimental Animal Center, College of Medicine, National Taiwan University, body weight 250–330 g, were anesthetized and their livers were perfused in situ with cooled normal saline via portal vein cannula to exsanguinate them. A 25% (w/v) liver homogenate was prepared in ice-cold Tris-HCl buffer, pH 7.4, using a Teflon-glass homogenizer (Glas-Col, Terre Haute, IN) and centrifuged at 480 × g for 10 min. The supernatant was taken for the experiment. Mitochondria and microsomal fractions were collected by further differential centrifugation. Precipitation at 9000 × g, 20 min (Kubota JA-21) for mitochondria and precipitation at 105000 × g, 60 min (Beckman L8-M Ultra-centrifuge) for microsome were obtained. The pellets were resuspended in 50 mM Tris-HCl buffer pH 7.4 to make up half the original volume so that the microsome or mitochondria content was equivalent to that in a 50% liver homogenate. All procedures were performed at 0–4°C. Homogenate and mitochondria were used immediately after preparation. Microsome was pooled and stored under –80°C until use. Protein concentration was determined by the Lowry method.

Metabolic Reaction

The 50 mM Tris-HCl buffer pH 7.4 was used as solvent for preparation of UDPGA (20–100 mM), MgCl2 (50 mM) and

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VPA (10–600 ug/ml) solutions. Triton-X 100 was added to homogenate, mitochondria or microsome suspension (10 ul/6 ml). Incubations were carried out in 1.0 ml total volume. Test tubes containing 0.2 ml liver homogenate, mitochondria or microsome, with 0.1 ml UDPGA, 0.1 ml MgCl₂ and 0.1 ml Tris-HCl buffer pH 7.4 solutions were preincubated in a shaking water bath at 37°C for 3 min, then 0.5 ml VPA solution (37°C) was added to the reaction mixture to start reaction at 37°C. After 2, 5, 10 or 15 min the reaction was stopped by adding 0.5 ml of 4 N H₂SO₄. Samples were taken to determine VPA-G and protein concentrations.

Assay of VPA and Its Glucuronide Metabolite

The concentration of VPA was determined by gas-chromatography (GC) (10). VPA-G was hydrolyzed to VPA and determined by GC (11). Briefly, VPA in the reaction mixture was extracted under acidic condition with chloroform for GC analysis. The aqueous layer remaining after removal of free VPA was washed once again with chloroform to assure complete removal of free VPA. To the aqueous layer was added molar excess of β-glucuronidase and incubated in a shaking water bath (80°C) for hydrolysis of VPA-G to VPA.

Estimation of Apparent Kinetic Constants for Glucuronide Conjugation

The conjugation rate (v₀) was estimated by linear regression of VPA-G concentration-time curve. The V_max and the K_m values for the formation of VPA-G were determined by Michaelis-Menten equation (eq. 1):

\[ v_0 = \frac{V_{\text{max}} \cdot C_o}{(K_m + C_o)} \]  

(1)

where C_o is the initial concentration of VPA in the reaction solution. Regression was performed by PCNONLIN (12) with the initial data estimated by Lineweaver-Burk plot.

Calculation of Hepatic Glucuronide Conjugation Clearance (CL_G)

Applying the values of metabolic kinetic parameters corrected to the body weight basis (by equation 2) and the data of steady-state unbound plasma concentration (C_uxs) of VPA from a previous study (8) into equation 3, CL_G of VPA in vivo was estimated.

\[ v_{\text{max}} \text{ per kg animal} = \left( \frac{v_{\text{max}} \text{ per ml reaction solution}}{\text{liver concentration in the reaction solution (g/ml)}} \right) \times \text{liver weight (g) per kg animal.} \]  

(2)

\[ \text{CL}_G = \frac{v_0}{C_{\text{uxs}}} = \frac{v_{\text{max}}}{(K_m + C_{\text{uxs}})} \]  

(3)

RESULTS

The Optimal Condition for the Experiment

An extended drug concentration range covering the clinical therapeutic unbound plasma level of VPA was studied. Different concentrations of liver homogenate, mitochondria or microsome were tested for detectability and linearity of VPA-G formation. The concentrations of 5% liver homogenate and 10% microsome in the final reaction media produced suitable results for this experiment; and the cofactor UDPGA was added in large excess to the substrate VPA. The formation of VPA-G was linear up to 15 min (Fig. 1). Presence of Triton-X 100 minimized the scattered results of the VPA-G formation rate.

VPA-G Formation in Liver Homogenate and in Microsomal Fraction

The VPA-G formation rate (v_0) for various initial concentrations (C_o) of VPA is shown in Fig. 2. The Lineeweaver-Burke plots demonstrated a single enzyme catalyzed process. The plot of v_0 versus C_o reveals a typical Michaelis-Menten type. The metabolic parameters estimated from the 5% liver homogenate reaction mixture are: V_max = 2.3 ± 0.2 (SE) nmole/min/ml (0.41 nmol/min/mg protein) and K_m = 313.8 ± 56 nmole/ml, and that from the 10% microsome reaction mixture are: V_max = 3.1 ± 0.31 (SE) nmole/min/ml (0.48 nmol/min/mg microsomal protein) and K_m = 161.0 ± 52 nmole/ml, respectively (Table 1). Liver weight of a guinea pig, 10–14 g, is equivalent to 4.1% body weight.

VPA-G Formation in Mitochondria Fraction

VPA-G was not detectable after incubation of VPA with 10–50% mitochondria suspension as the process described.

![Fig. 1](image1.png)

![Fig. 2](image2.png)