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 (V_{max}) and Michaelis-Menten constant (K_m) of VPA were determined.

Results. On a body weight basis, the V_{max} and the K_m 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 anticonvulsant. 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 K_m 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), $MgCl_2$ (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 $MgCl_2$ 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 H_2SO_4. 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 (v_o) 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):

$$V_o = V_{max} * C_o/(K_m + C_o) \quad (1)$$

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_{uss}) of VPA from a previous study (8) into equation 3, CL_G of VPA in vivo was estimated.

$$v_{max} \text{ per kg animal} = [v_{max} \text{ per ml reaction solution} / \text{liver concentration in the reaction solution (g/ml)}] \times \text{liver weight (g) per kg animal.} \quad (2)$$

$$CL_G = v_o/C_{uss} = v_{max}/(k_m + c_{uss}) \quad (3)$$

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_o) for various initial concentrations (C_o) of VPA is shown in Fig. 2. The Lineveaver-Burke plots demonstrated a single enzyme catalyzed process. The plot of v_o versus C_o reveals a typical Michaelis-Menten type. The metabolic parameters estimated from the 5% liver homogenate reaction mixture are: $V_{max} = 2.3 \pm 0.2$ (SE) nmole/min/ml (0.41 nmol/min/mg protein) and $K_m = 313.8 \pm 56$ nmole/ml, and that from the 10% microsome reaction mixture are: $V_{max} = 3.1 \pm 0.31$ (SE) nmole/min/ml (0.48 nmol/min/mg microsomal protein) and $K_m = 161.0 \pm 52$ 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.

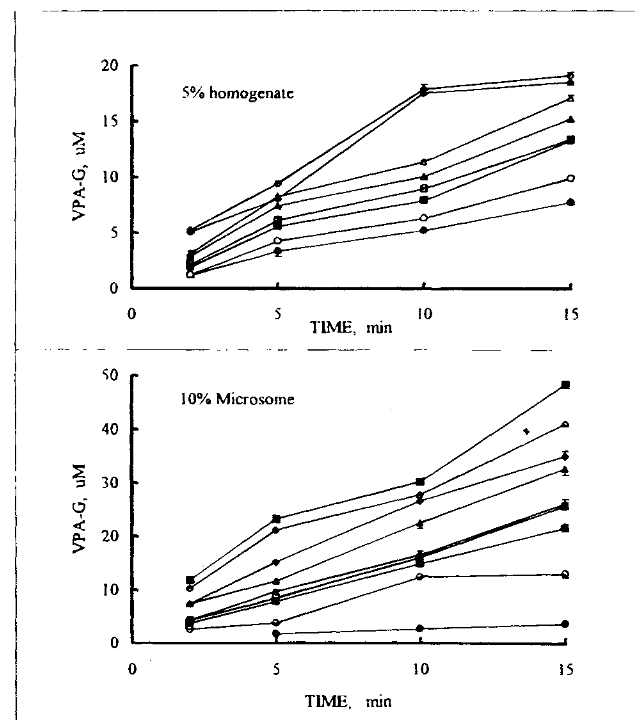

Fig. 1. The glucuronide conjugation rate of valproate in 5% liver homogenate and 10% hepatic microsome preparation of guinea pigs. Lines represent different initial concentrations of valproate, from the top to the bottom: 200, 150, 75, 50, 40, 30, 20, 10, and 5 ug/ml. In 5% liver homogenate the reaction of 5 ug/ml was not detectable. Each point represents mean with SE bar of three determinations. Some SE bars are within the points.

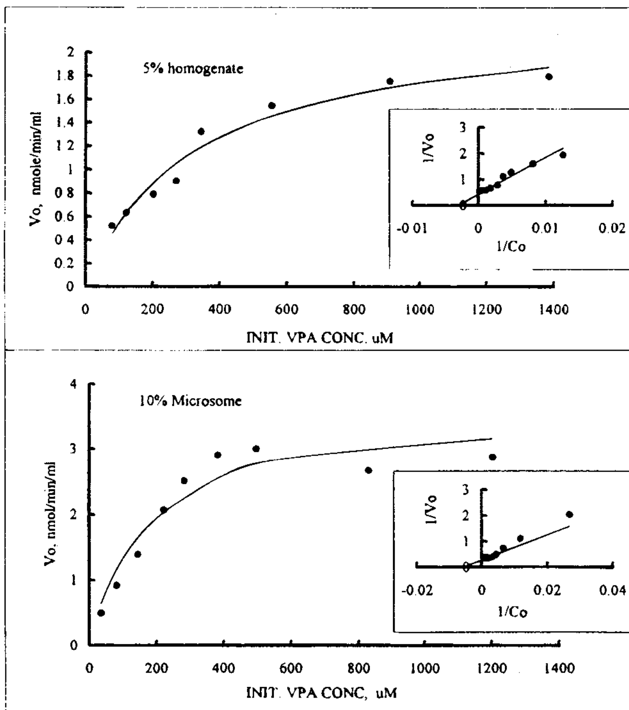

Fig. 2. The glucuronidation rate (Vo) vs initial concentration (Co) of valpraote in 5% liver homogenate and in 10% microsome preparation of guinea pigs. Insets are Lineweaver-Burke plot.

Hepatic Glucuronide Conjugation Clearance (CL_G)

CL_G of VPA in vivo is shown in Table 2. The CL_G demonstrated an obvious decrease (more than one half, from 3.46 to 1.66 ml/min/kg) as the C_{uss} exceeded K_m value to approach V_{max}. At clinical plasma level the glucuronide conjugation of VPA contributed 0.28–0.46 fraction to its total elimination.

DISCUSSION

A previous study in guinea pigs estimating the metabolic parameters of VPA from *in vivo* plasma elimination obtained biphasic data (8). Further analysis of conjugation metabolite concentrations of VPA in liver found that the primary parameters, V_{max1} = 1.52 umole/min/kg and K_{m1} = 0.15 umole/ml,

Table 1. Glucuronidation Rate Constants of VPA in Guinea-pigs

	in vitro		
	5% liver homog.	10% microsome susp.	in vivo (8)
Vmax			
nmol/min/ml reac.	2.3 (0.2)[a]	3.1 (0.31)	
nmol/min/mg prot.	0.4 (0.02)	0.5 (0.08)	
μmol/min/kg	1.8	1.2	1.5 (0.40)
K_m			
μmol/ml	0.31 (0.06)	0.16 (0.05)	0.15 (0.08)
CL_{intG} (= Vmax/K_m)			
ml/min/kg	6	7.5	7.5

[a] Data in parentheses are the standard errors of nonlinear estimation.

Table 2. Glucuronide Conjugation Clearance (CL_G) of Valproate

C_{uss} (8), μmol/ml	0.02	0.08	0.22	0.79	1.34	3.94	8.58
CL_u (8), ml/min/kg	16.03	16.89	7.51	4.58	3.87	2.31	1.55
CL_G[a], ml/min/kg							
A	5.63	4.74	3.46	1.66	1.10	0.42	0.20
B	6.67	6.25	3.95	1.26	0.80	0.29	0.14
C	6.82	5.36	3.57	1.52	0.97	0.36	0.17
CL_G/CL_u							
A	0.35	0.28	0.46	0.36	0.28	0.18	0.13
B	0.42	0.37	0.53	0.28	0.21	0.13	0.09
C	0.43	0.32	0.48	0.33	0.25	0.16	0.11

[a] Based on the V_{max} and K_m values obtained from liver homogenate (part A), liver microsome suspension (part B) and plasma elimination (8) (part C).

are most likely the conjugation metabolic rate (8). The K_m value, 0.3 (liver homogenate) or 0.16 (microsome suspension) umole/ml, obtained from the present experiment is comparable with the K_{m1} obtained from previous *in vivo* study. The V_{max} measured from the present study and corrected to the body weight basis is again comparable with the V_{max1} value of the *in vivo* results (Table 1). The intrinsic clearance of glucuronidation (CL_{intG}) estimated from *in vitro* and *in vivo* is also equivalent (Table 1). The CL_G estimated from the present in vitro metabolic parameters contributes 0.28–0.46 fraction of total clearance of unbound VPA (CL_u) (8) at clinical plasma levels, which is in agreement with the dose fraction of VPA-G metabolite observed in rats (9,11) and in a clinical study (0.23–0.46) (14).

The K_m value is at clinical middle concentration of unbound VPA (13). Consequently, the glucuronidation of VPA should be nonlinear within clinical concentration range because Michaelis-Menten equation shows the most pronounced nonlinearity around K_m. At lower concentration where Co $<<<$ K_m, glucuronidation rate will increase proportionally with the increasing concentration ($v_o = (V_{max}/K_m) \times C_o$); and at higher concentration where Co $>>>$ K_m, glucuronidation will not increase proportionally with increasing concentration but approach to a constant, V_{max}. Consequently, the effect of VPA on its own metabolic rate, or dose-dependent metabolism of VPA observed simply from VPA-G-to-dose ratios should lead to inconsistent conclusions when data were obtained from different plasma concentration regions between studies. The K_m and V_{max} obtained from the *in vitro* study can adequately interpret the dose-dependent metabolism of VPA observed from a human study (15) that the excretion rate of VPA-G during the initial period after high doses of VPA was not related to dose, and a large portion of VPA-G was excreted during that period. Such phenomenon is rational according to our results that the plasma unbound VPA concentration during the initial period after such high doses must be much higher than the K_m value, and the v_o approaches V_{max} (not related to dose or concentration). Increase of VPA-G excretion in patients (14) and in rats (6) after chronic treatment has been reported. The mechanism may also include nonlinear metabolic rate. The plasma concentration after a single initial dose is usually much lower than that at steady-state, and therefore, glucuronidation rate should be different in spite of equivalent dose. These results imply that the glucuronidation rate in human may also be nonlinear within clinical concentration range, and that the glucuronidation kinetic parameters are quite similar between human and guinea pigs or rats.

In conclusion, the glucuronide conjugation is the primary metabolic pathway of VPA, and is nonlinear and nearly saturable at clinical concentration level. Measurement of VPA-G at different plasma concentration level without consideration of nonlinear metabolic rate may lead to different conclusion. Prediction of *in vivo* glucuronidation rates from experimental metabolic rate parameters is feasible.

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