EFFECT OF N-(2-PROPYLPENTANOYL) UREA ON RAT HEPATIC DRUG METABOLIZING ENZYMES

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ABSTRACT

Effect of N-(2-propylpentanoyl)urea (VPU) on rat hepatic drug metabolizing enzymes was investigated. Median effective dose of VPU as well as that of valproic acid (VPA, the prototype of VPU) were given intraperitoneally to male Wistar rats for 7 days. On the day after, the animals were sacrificed by cervical dislocation. Hepatic microsomal and cytosolic subfractions were isolated. Microsomal total cytochrome P450 contents, microsomal cytochrome P450 enzyme-substrate activities, cytosolic glutathione S-transferase activities and hepatic total glutathione were determined. No effects of VPU and VPA were observed on total cytochrome P450 contents, ethoxy- and methoxyresorufin O-dealkylase activities (representing CYP 1A1 & 1A2 activities), aniline 4-hydroxylase activities (representing CYP 2E1 activities), glutathione S-transferase activities and hepatic total glutathione. However, pentoxy- and benzyloxyresorufin O-dealkylase activities (representing CYP 2B1 & 2B2 activities) were significantly increased by VPU. Correspondingly, CYP 2B1 & 2B2 proteins detected by Western blotting were slightly increased following VPU treatment. Further study on the effect of VPU on other isoforms of CYP, involving in human drug metabolism, was suggested.

Key words: N-(2-propylpentanoyl) urea, valproic acid, cytochrome P450, glutathione S-transferase, total glutathione

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INTRODUCTION

Valproic acid (VPA) (Figure 1), a conventional widely used anticonvulsant drug, has been known for its moderate efficacy as well as a number of side effects[1-3]. Hepatotoxicity and teratogenicity are two major drawbacks of this drug reported in both experimental animals and humans[2-4]. Therefore, several laboratories have attempted to develop improved derivatives so as to reduce these unwanted effects but improve the efficacy[5]. N-(2-propylpentanoyl) urea or valproyl urea (VPU) (Figure 2) is a monoureide analogue of VPA which was synthesized in 1992 in the laboratory of the Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University[6].

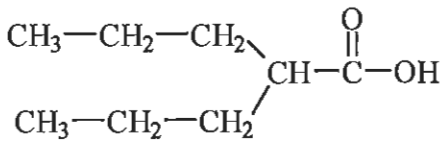

Figure 1 Valproic acid (VPA)

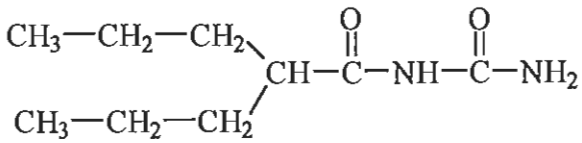

Figure 2 N-(2-propylpentanoyl) urea or valproyl urea (VPU)

VPU possessed a greater anticonvulsant activity compared with its parent compound, VPA, in different animal models tested either in protection against maximal electroshock seizure or pentylenetetrazol-induced convulsion. In addition, VPU demonstrated a greater margin of safety while produced less neurological impairment than did VPA[7]. Developmental toxicity, regarding effects on axial rotation and embryonic growth, was lower in VPU-treated animals compared with those of VPA-treated[8]. Hepatotoxic effects were observed *in vivo* and *in vitro* only at large dose of VPU administration[9]. Pharmacokinetic studies utilizing ^{14}C-VPU and autoradiographic technique demonstrated a rapid distribution characteristic of VPU into various organ tissues. In addition, *in vitro* studies using carboxylesterase from human liver and phenobarbital-treated mice liver showed that VPU was negligibly hydrolysed into VPA. Therefore, it was postulated that VPU *per se* and/or any metabolites other than VPA was responsible for the anticonvulsant activity[10].

Anticonvulsant drugs are associated with a wide range of drug interactions, including hepatic enzyme induction and inhibition. Phenobabital, primidone, phenytoin and carbamazepines are reported to be hepatic enzyme inducers whereas valproic acid is an enzyme inhibitor[11]. Preliminary study of the effect of VPU on hepatic drug metabolizing enzymes demonstrated a prolong barbiturate sleeping time[7].

In addition, *in vitro* study using human liver microsome, VPU demonstrated an inhibitory effect on CYP 2C9 & CYP 1A2[10]. Therefore, the aim of this study was to assess the effect of VPU on phase I metabolizing enzymes, cytochrome P450 (CYP) including CYP 1A1, CYP 1A2, CYP 2B1, CYP 2B2 and CYP 2E1. Effect of VPU on glutathione S-transferase (GST), an important phase II metabolizing enzymes, as well as its effect on hepatic total glutathione (GSH) were also evaluated.

MATERIALS AND METHODS

Materials

The following chemicals were obtained from Sigma Chemical Co. (USA): 4-aminophenol, aniline hydrochloride, bovine serum albumin, 1-chloro-2, 4-dinitrobenzene, cupric sulfate, 5,5′-dithiobis-(2-nitrobenzoic acid), ethylenediamine tetraacetic acid, Folin & Ciocalteu's phenol reagent, glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PD), glutathione (reduced form), nicotinamide adenine dinucleotide phosphate (NADP), potassium phosphate, potassium phosphate monobasic anhydrous, sodium carbonate, sodium citrate, sodium phosphate dibasic anhydrous, Trisma® base, valproic acid, benzyloxyresorufin, ethoxyresorufin, methoxyresorufin, pentoxyresorufin and resorufin. Magnesium

chloride, phenol, potassium chloride, sodium chloride, sodium hydroxide and trichloroacetic acid were purchased from E. Merck (Germany). Absolute ethanol and glycerol were obtained from Carlo Erba (USA). Methanol (HPLC grade) was obtained from BDH Laboratory Supplies (England). Sodium dithionite was purchased from Fluka Chemic (Japan). Polyethylene glycol (PEG) 400 was purchased from T. Chemical Ltd. Partnership (Thailand). VPU was obtained from Dr. Chamnan Patarapanich. All materials used in Western blot analysis were generously obtained from Prof. Kan Chiba.

Animals

Adult male Wistar rats, weighing 250-300 gm, were obtained from National Laboratory Animal Centre, Mahidol University, Salaya, Nakornpathom. The animals were housed in animal care facilities at the Faculty of Pharmaceutical Sciences, Chulalongkorn University and acclimatized for at least 1 week before the experimentation.

Experimental model

Twenty-four male Wistar albino rats composed 4 treatment groups: control group 1, control group 2, VPA treatment group and VPU treatment group. Four animals were studied simultaneously during each experimental period (1 animal/each treatment group). Animals in control group 1 were given sterile water for injection, ip, for 7 days whereas PEG 400 was given to animals in control group 2 in the same manner. Animals in VPA treatment group received VPA at a dosage of 250 mg/kg/day, ip, for 7 days. VPU, suspended in PEG 400, were given to animals in VPU treatment group at a dosage of 80 mg/kg/day in the same manner.

On the day after the dosing, the animals were sacrificed by cervical dislocation. Livers were perfused *in situ* with ice-cold 0.9 % sodium chloride via portal vein. Small portion of livers were stored at – 80°C for total GSH assay. Microsomal and cytosolic subfractions were prepared by homogenizing the remaining portion of livers in 3 volume of phosphate buffer, pH 7.4. After the homogenate were centrifuged at 4°C for 30 minutes at 10,000 g, the obtained supernatants were further centrifuged at 4°C for 60 minutes at 100,000 g. The microsomal pellets were resuspended in 5 ml of phosphate buffer pH 7.4 containing 20% v/v glycerol. Microsomal and cytosolic subfractions were stored at – 80°C. Liver microsomal and cytosolic protein concentrations were determined by the method of Lowry et al.[12].

Total CYP content determination

Total CYP contents in microsomal subfractions were determined spectrophotometrically by the method of Omura and Sato[13]. The quantity of CYP was calculated from the absorbance difference (450-490 nm) after reduced by sodium dithionite and bubbled with carbon monoxide. The extinction coefficient of 91 mM^{-1} cm^{-1} was used for a calculation.

Alkoxyresorufin O-dealkylation assay

The O-dealkylations of ethoxy-, methoxy, benzyloxy- and pentoxyresorufin by liver microsomes were determined according to the method of Burke and Mayer[14] and Lubet et al.[15] with slight modifications. Each 1 ml of reaction mixture contained 0.1 M Tris buffer, pH 7.4, alkoxyresorufin (5 µM), NADPH regenerating system [comprising NADP (1 mM), G6P (5mM), and magnesium chloride (3 mM)], and microsomal sample (containing 100 µg of protein). Three tubes of 1 ml of reaction mixture were prepared for each sample (1 tube for a blank and the remaining 2 tubes for a sample). The reaction was started by the addition of 10 µl of G6PD (100 units/ml) in 20 mM potassium phosphate buffer, pH 7.4 after a 2 minutes preincubation. Ten microlitre of 20 mM potassium phosphate buffer, pH 7.4 was used in placed of G6PD in the blank. After 5 minutes of incubation at 37°C, the reaction was terminated by adding 1 ml of methanol (HPLC grade).

The O-dealkylations of alkoxyresorufins were determined by measuring the amount of resorufin formed by fluorescence spectrophotometer (excitation λ = 556 nm and emission λ = 589 nm) and expressed as a function of time and amount of protein.

Aniline 4-hydroxylation assay

The 4-hydroxylation of aniline by liver microsomes was determined according to the method of Schenkman et al.[16], utilizing aniline hydrochloride as a substrate. The reaction was determined by measuring the amount of a metabolite, 4-aminophenol, by

spectrophotometer at 630 nm and expressed as a function of time and amount of protein.

Glutathione S-transferase assay

Liver cytosolic GST activities were determined spectrophotometrically at 340 nm according to the method of Warholm et al.[17], utilizing 1-chloro-2,4-dinitrobenzyene (CDNB) as a substrate. The extinction coefficient of 9.6 mM^{-1} cm^{-1} was used for a calculation of enzyme activities which were expressed as a function of time and amount of protein.

Total glutathione determination

Total GSH concentrations were determined by homogenizing the liver samples with 0.1 M Tris buffer, pH 7.4 and precipitating protein with 10% trichloroacetic acid. After centrifugation at 5,000 g for 10 minutes, the supernatants were then examined for total GSH according to the method of Sedlak and Lindsey[18], utilizing 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as a substrate. Total GSH was determined by measuring amount of a product, 2-nitro-5-thiobenzoic acid, by spectrophotometer at 412 nm and expressed as a function of liver protein or weight of liver.

Western blot analysis

Liver microsomes (15 µg of protein) were subjected to sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli[19] and the proteins were transferred onto a nitrocellulose membrane according to the method of Towbin et al.[20] with a minor modifications[21]. Rabbit antibody to rat CYP 2B1 & 2B2 was used as the first antibody whereas goat anti-rabbit IgG with rabbit peroxidase-antiperoxidase complex was used as the second antibody. The visualized stain was developed by adding a fresh solution of 3,3' diaminobenzidine and hydrogen peroxide. The intensities of the immunoblot were measured with an Epson GT-9600 scanner equipped with NIH Image/Gel Analysis Program adapted for Macintosh computers.

Statistics

One-way analysis of variance (ANOVA) was used to evaluate the mean differences among 4 treatment groups. If the differences were significant, Student-Newman-Keuls tests were used for pairwise comparisons. In all cases, the criteria for statistical significance were $p < 0.05$.

RESULTS

Effect of VPU on hepatic microsomal total CYP content

PEG 400, which was used as a solvent of VPU, exhibited no effects on total CYP content. No significant effects of both VPU and VPA on total CYP content were found as compared to those in the control group 1 & 2 (Table 1).

Table 1 Effect of VPU on hepatic microsomal total CYP content (mean ± SE)

Treatment group	Total CYP content [a] (n =6)
1. Control group 1	0.620 ± 0.031
2. Control group 2	0.639 ± 0.031
3. VPA treatment group	0.534 ± 0.026
4. VPU treatment group	0.621 ± 0.031

[a] Unit expressed as nmole/mg protein

Effect of VPU on hepatic microsomal alkoxyresorufin O-dealkylase activities

No effects of PEG 400 were observed on the O-dealkylation of all alkoxyresorufins used in this study. Both ethoxy- and methoxyresorufin O-dealkylase (EROD and MROD, respectively) activities which represented CYP 1 A1 & 1 A2 activities, demonstrated no change following VPU and VPA treatments as compared to those in control group 1 & 2. Benzyloxy- and pentoxyresorufin O-dealkylase (BROD and PROD, respectively) activities, which represented CYP 2B1 & 2B2 activities, showed no change after VPA treatment. Significant increases of BROD and PROD activities were observed following VPU treatment as compared to those in control group 1 & 2 (Table 2). Corresponding to the activity data, CYP 2B1 & 2B2 proteins detected by Western blotting were slightly increased following VPU treatment (Figure 3).

Table 2 Effect of VPU on hepatic microsomal alkoxyresorufin O-dealkylase activities (mean ± SE)

Treatment group	Alkoxyresorufin O-dealkylase activities [(a)]			
	EROD (n =6)	MROD (n =6)	BROD (n =6)	PROD (n =6)
1. Control group 1	60.67 ± 8.27	22.00 ± 3.76	28.67 ± 4.81	5.00 ± 0.86
2. Control group 2	58.33 ± 8.07	14.33 ± 2.80	27.00 ± 4.73	2.33 ± 1.31
3. VPA treatment group	57.33 ± 10.46	19.33 ± 3.68	28.67 ± 8.89	3.00 ± 1.44
4. VPU treatment group	70.33 ± 7.14	20.00 ± 3.58	68.33 ± 10.20*	9.67 ± 0.95*

[(a)] Unit expressed as pmole/mg protein/min
* $p < 0.05$; VPU vs Control group 1 & 2

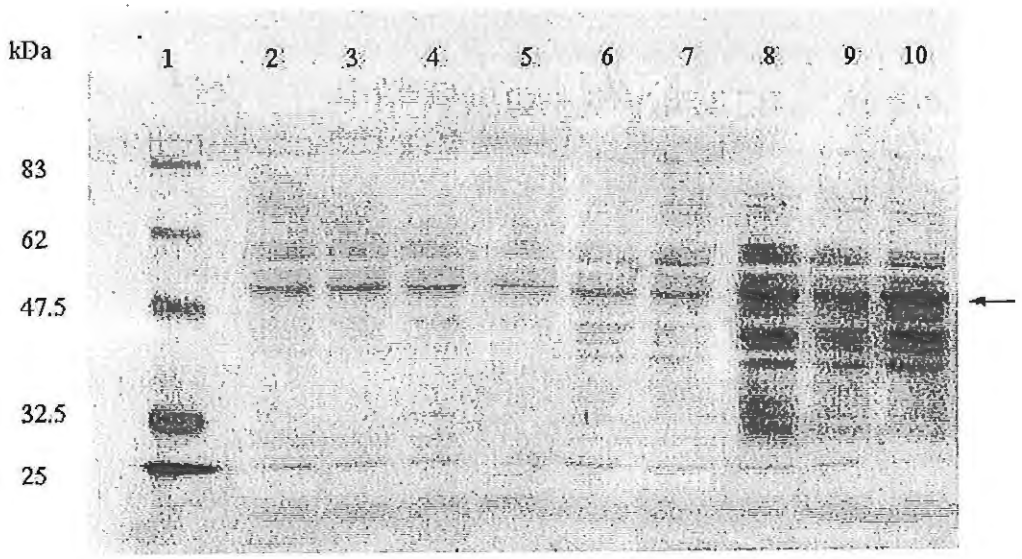

Figure 3 Western immunoblot of liver microsomes isolated from rats treated with PEG400 (lane 2-4), VPA (lane 5-7) and VPU (lane 8-10) as described under materials and methods. Protein marker was shown in lane 1. The arrow indicated CYP 2B1 and 2B2

Effect of VPU on hepatic microsomal aniline 4-hydroxylase activity

No effect of PEG 400 was observed on hepatic microsomal aniline 4-hydrolase activity, which represented CYP 2E1 activity. Likewise, both VPU and VPA exhibited no effect on this enzyme activity as compared to those in control group 1 & 2 (Table 3).

Effect of VPU on hepatic cytosolic GST activities

No effect of PEG 400 was observed on hepatic cytosolic GST activities. Likewise, both VPU and VPA treatments exhibited no effect on these enzyme activities as compared to control group 1 & 2 (Table 4).

Effect of VPU on hepatic total GSH concentration

PEG 400 exhibited no effect on total GSH concentration. Similarly, both VPU and VPA treatments demonstrated no effect on total GSH concentration as compared to those in control group 1 & 2 (Table 5).

Table 3 Effect of VPU on hepatic microsomal aniline 4-hydroxylase activity (mean ± SE)

Treatment group	Aniline 4-hydroxylase activity [a] (n =6)
1. Control group 1	0.259 ± 0.017
2. Control group 2	0.295 ± 0.029
3. VPA treatment group	0.210 ± 0.015
4. VPU treatment group	0.272 ± 0.028

[a] Unit expressed as nmole/mg protein/min

Table 4 Effect of VPU on hepatic cytosolic GST activities (mean ± SE)

Treatment group	GST activities [a] (n =6)
1. Control group 1	919.08 ± 111.84
2. Control group 2	996.00 ± 97.06
3. VPA treatment group	969.20 ± 95.27
4. VPU treatment group	889.24 ± 74.89

[a] Unit expressed as nmole/mg protein/min

Table 5 Effect of VPU on hepatic total GSH concentration (mean ± SE)

Treatment group	total GSH concentration	
	nmole/mg protein (n =6)	μmole/g liver (n =6)
1. Control group 1	30.25 ± 2.02	6.62 ± 0.43
2. Control group 2	30.17 ± 0.69	6.52 ± 0.31
3. VPA treatment group	34.77 ± 1.45	7.01 ± 0.20
4. VPU treatment group	34.19 ± 1.78	7.14 ± 0.24

DISCUSSION

Therapy with antiepileptic drugs has been known to be associated with a wide range of drug interactions, including hepatic enzyme induction and inhibition as well as protein-binding displacement. Phenobarbital, primidone, phenytoin and carbamazepine are inducers of CYP 2C and CYP 3A whereas valproic acid is inhibitors of CYP 2C, UDPGT and epoxide hydroxylase[11]. Therefore, in the drug development process of a new antiepileptic drug, preclinical testing involving effect of that compound on hepatic drug metabolizing enzymes is recommended[22]. In this study, we followed the protocol of the Anticonvulsant Screening Project (ASP) of the Antiepileptic Drug Development (ADD) program that suggested several hepatic parameter measurements following 7 days of a compound dosing. Dosages of both VPU and VPA used in this study were median effective doses (ED_{50}) protected rats against maximal electroshock convulsion which were 80 mg/kg and 250 mg/kg, respectively[7].

In this study, we evaluated the effect of VPU on the activities of four alkoxyresorufin O-dealkylase, including EROD and MROD which represented activities of CYP 1A1 & 1A2, as well as BROD and PROD which represented activities of CYP 2B1 & 2B2. Besides these major inducible isoforms of CYP, we examined the effect of VPU on aniline 4-hydroxylase activity which represented another important inducible isoform of CYP, CYP 2E1. No effect of PEG 400, which used as a solvent of VPU, was observed on any parameters measured in the study. VPU and VPA treatments did not affect hepatic microsomal total CYP content. However, significant increases in BROD and PROD activities were observed following VPU treatment. Because the induction of CYP 2B1 & 2B2 activities by VPU was much less than that by phenobarbital, the corresponding CYP 2B1 & 2B2 proteins detected by Western blotting were shown slightly increased. This was probably due to the rapid excretion of VPU from the body[10]. Even though the induction effect of VPU on CYP 2B1 & 2B2 existed, the effect might be too subtle to affect total CYP content which was detected by the moderate sensitive method. *In vitro* study utilizing rat hepatocyte cell culture, VPA demonstrated an induction effect on CYP 2B1 & 2B2[23]. However, the effect was not shown in the *in vivo* study when multiple dosing of VPA were administered to the animals[23] possibly due to the short half-life of this drug in rats (10-20 minutes)[24]. Similarly, VPA did not show an induction effect on CYP 2B1 & 2B2 in our *in vivo* study.

The induction effect of VPU on CYP 2B1 & 2B2 found in this study could not explain the prolong barbiturate sleeping time after simultaneously single administration of VPU and pentobarbital reported previously[7]. In this regard, VPU might act as an enzyme inhibitor of CYP 2B1 & 2B2 by competitively competing with pentobarbital binding to the same binding site on these isoforms. Dual effects of VPU on CYP 2B1 & 2B2 might be possible; an induction effect following multiple doses but an inhibition effect following a single dose of this compound. Further study on the latter effect should be elucidated.

CYP 1A1 & 1A2, presenting both in humans and rats at low level, are toxicologically important because they generally convert environmental chemicals (e.g. aromatic and heterocyclic hydrocarbons) and natural compounds (e.g. aflatoxin B_1) to toxic metabolites. Such metabolic activation is thus the most frequent mechanism of transformation of procarcinogen to ultimate carcinogen or carcinogenic intermediates. Therefore, these enzymes normally play a key role in carcinogen activation. Likewise, CYP 2E1 also bioactivates a number of compounds (e.g. acetaminophen), to yield cytotoxic or carcinogenic intermediates[25-26]. Thus the induction of CYP 1A1 & 1A2 and CYP 2E1 isoforms generally increase the risk of toxicological consequences following exposure to environmental chemicals or other xenobiotics which are bioactivated by these enzymes. The present results demonstrated that both VPU and VPA, at a median effective dose, did not have any effects on CYP 1A1 & 1A2 and CYP 2E1 activities. This should be an advantage feature of both VPU and VPA regarding a potential increase risk of toxicity of other xenobiotics.

Our results did not show any effects of VPU on hepatic cytosolic GST activities and total GSH. Large dose of VPU was reported to produce a depletion of hepatic total GSH and hepatotoxic effects[9]. This indicated that VPU, administered at a median effective dose using in this study, was safe regarding the reactive metabolites as well as hepatotoxicity (data was not shown).

In summary, median effective dose of VPU administered to male Wistar rats for 7 days demonstrated no effects on hepatic

microsomal CYP 1A1 & 1A2, CYP 2E1 activities, hepatic cytosolic GST activities and hepatic total GSH CYP 2B1 & 2B2 activities were significantly increased following VPU treatment, correspondingly to a slight increase of CYP 2B1 & 2B2 proteins detected by Western blotting. Effect of VPU on other isoforms of CYP, involving in human drug metabolisms, was suggested for a further study.

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