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SHORT COMMUNICATIONS

Valproate glucuronidation by rat liver microsomes. Interaction with parahydroxyphenobarbital

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Sodium valproate (VPA), a branched short-chain fatty acid, is commonly used in the treatment of epilepsy, either alone or in combination with other drugs [1]. Several interactions have been described, one of which involves a valproate-induced increase in phenobarbital (PB) blood levels [2, 3]. This interaction is of clinical significance, since various side effects are related to high PB blood concentrations.

In an attempt to elucidate the mechanism of the interaction between VPA and PB, pharmacokinetic studies were previously set up in normal subjects [4] and in epileptic patients [5], and it was suggested that PB metabolism could be inhibited by VPA. The major identified metabolite of PB is the hydroxylated derivative in the paraposition, parahydroxyphenobarbital (POH PB); part of this metabolite is glucuronidated before elimination via the kidney [6, 7].

In rat hepatic microsomes, PB hydroxylation has been shown to be inhibited by valproate [8], but this cannot explain the decreased ratio of conjugated POH PB over total POH PB excreted in urine during PB–VPA therapy [5, 9, 10]. Valproate is extensively metabolized in man, and a significant portion of the metabolism occurs via conjugation with glucuronic acid [2]. Thus, glucuronidation is a common metabolic pathway of both drugs.

To our knowledge, the kinetics of valproate glucuronidation has not been previously described. The primary purpose of this study was to investigate the kinetics of UDP glucuronyltransferase (UDP GT)—EC 2.4.1.17—towards valproate in rat liver microsomes, and to study modifications in the enzymes activity when both VPA and POH PB were used as substrates.

Materials and methods

Chemicals. Phenobarbital sodium salt was from the Cooperative Pharmaceutique Française (Melun, France). Parahydroxyphenobarbital was synthesized by Pharmacie Centrale des Hôpitaux (Paris, France).

Valproic acid sodium salt was kindly donated by Labaz (Ambarès, France). UDP glucuronic acid, sodium salt and Triton X-100 were from Sigma (St. Louis, MO, U.S.A.).

Treatment of animals and preparation of liver microsomes. Male Wistar rats were fed a commercial diet. When specified, phenobarbital sodium salt was dissolved in distilled water and given once a day for 3 days (100 mg/kg/day) intraperitoneally.

Microsomes were prepared as previously described [11]. Briefly, the livers were quickly excised at 4°, weighed, minced and homogenized with an ice-coated Braun glass and Teflon homogenizer in a medium containing 20 mM Tris HCl pH 7.4, 1 mM EDTA and 1.15% KCl. Unbroken cells, nuclei and mitochondria were removed by centrifuging the homogenates at 9000 *g* for 20 min at 5°. The resulting supernatants were spun at 105,000 *g* for 60 min. The pellet was resuspended in Tris medium and spun at 105,000 *g* for 60 min. The microsomal pellet was resuspended in Tris medium at a concentration of 70 mg of microsomal protein per ml. Microsomes were kept frozen (− 70°) until use, without significant loss of activity.

Proteins were determined according to the method of Lowry *et al.* [12] with bovine serum albumin as a standard; cytochrome P-450 was measured according to the method of Omura and Sato [13].

Assays of UDP glucuronyltransferase. Unless otherwise specified, enzyme activity towards valproate and POH PB was assayed with aglycone concentrations of 1 mM and 0.25 mM respectively. VPA was dissolved in distilled water and POH PB in a minimum volume of methanol (10 μl); for kinetic studies, aglycone concentrations ranged from 0.1 to 1 mM.

Assays were performed at 37° in the presence of 20 mM Tris HCl pH 7.4, $MgCl_2$ 10 mM, UDP glucuronic acid 2 mM. In blanks, UDP glucuronic acid was omitted. Reactions were started by addition of activated microsomes; at least 20 min before assays, enzymes were fully activated with Triton X-100 added to give a detergent protein ratio of 0.10. Assays were performed under conditions leading to linear reaction rates with time (6 min) and protein concentration (5 mg per incubation, total incubation volume 0.5 ml).

The reaction was stopped by the addition of HCl 5 N (0.1 ml). Non-conjugated substrate was extracted and quantified by gas liquid chromatography as described below. Inclusion of a blank containing water instead of UDP glucuronic acid permitted subtraction of residual aglycone and calculation of the glucuronidated fraction. This procedure gave the same results as hydrolysis (β-glucuronidase or alkaline hydrolysis for VPA glucuronide, β-glucuronidase or acid hydrolysis for POH PB glucuronide) of the aqueous phase after removal of the excess of non-conjugated substrate.

Substrate analysis. Valproic acid was measured according to the same gas liquid chromatography method as described for plasma assay [14]; briefly, cyclohexane carboxylic acid (internal standard) was added to the incubation mixture at the end of the reaction; valproic acid and the internal standard were quantitatively extracted by 1 ml of chloroform. The organic phase was transferred and evaporated under a stream of nitrogen. The residue was dissolved in 50 μl of $CHCl_3$ and an aliquot injected into a 5% FFAP column.

POH PB was measured after ether extraction and flash methylation by gas liquid chromatography with a nitrogen phosphorus sensitive detector using the method described for measurement of PB in plasma [15]; heptabarbital was used as an internal standard.

Data analysis. Data were interpreted after construction of Lineweaver–Burk, Hofstee or Dixon plots. Regression analysis were performed by the least square method. Variance analysis was used as the statistical test.

Results and discussion

UDP glucuronyltransferase activity towards sodium valproate. Under our working conditions, UDP GT was fully activated with a Triton X-100 over microsomal protein ratio of 0.1. Furthermore, after addition of Triton X-100 to the microsomal preparation, the kinetics of the enzyme became linear on a Lineweaver–Burk plot (Fig. 1).

Apparent enzymatic parameters were calculated graphically from Hofstee plots: K_m and V_{max} were, respectively 0.65 ± 0.2 mM and 3.68 ± 0.78 nmol/min/mg protein

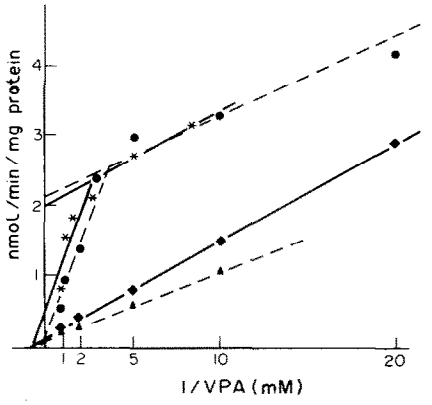

Fig. 1. Lineweaver–Burk plots of microsomal glucuronidation of sodium valproate. *, control native microsomes; ●, native microsomes from phenobarbital pretreated rats; ◆, control microsomes + Triton X-100; ▲, microsomes from phenobarbital pretreated rats + Triton X-100.

(mean ± S.E.M. of three experiments). Calculation of the Michaelis constant, K_m using the method of Cleland as described by Bock *et al.* [16] with various concentrations of UDP glucuronic acid (0.5–4 mM) and various concentrations of VPA (0.1–2 mM) gave similar results: K_m VPA 0.70 mM: K_m of UDP glucuronic acid calculated according to the same method was 1.95 mM.

After pretreatment with PB, UDPGT activity was enhanced 1.4-fold and compared to the cytochrome P-450 increase (Table 1); this induction could not be revealed on native microsome (Fig. 1). The K_m value was not significantly altered after pretreatment with PB (0.64 ± 0.21 mM), the maximum rate of the reaction V_{max} increased by approximately two-fold (7.18 ± 1.29 nmol/min/mg protein—mean ± S.E.M. of three experiments).

VPA glucuronidation by rat liver native microsomes shares the same properties as glucuronidation of other substrates: non-Michaelian kinetics [17] and an apparently non-inducible enzyme [18] with these two properties being modified when detergent (Triton X-100) is added to the microsomal proteins. The apparent K_m of the reaction was not significantly altered after pretreatment with PB; whereas the V_{max} was increased as described with other inducers and substrates [16]. A similar increase in UDPGT activity towards VPA after PB treatment was found by Watkins and Klaassen on rat microsomes activated with BRIJ 58, whereas 3-methylcholanthrene was unable to increase enzyme activity [19].

These results at least partially demonstrate some concordance between *in vitro* and *in vivo* data. In rats pretreated with PB, the percentage of conjugated VPA was significantly higher than in controls [19], which might be partly related to the increased amount of UDPGT after PB pretreatment. The same pattern was found in epileptic patients treated concomitantly with valproate and other antiepileptic drugs such as PB or phenytoïne: total body clearance of VPA was almost twice as high as in normal subjects receiving only VPA [20]. In humans, an average of 20% of the dose administered was recovered in urine as VPA glucuronide [2]. Thus, elimination through glucuronidation might account for an important part of total body clearance.

Since clinical studies have suggested the inhibitory effects of VPA (and/or one of its metabolite) in various drug metabolizing enzymes [3–5] we studied UDPGT activity towards VPA after pretreatment of rat with VPA itself. No effect was observed after 4 days of treatment (200 mg/kg/day) either on cytochrome P-450 level or on UDPGT activity (Table 1), thus corroborating previous studies showing that VPA, in contrast to other antiepileptic drugs, does not have any inducing properties [21, 22].

Interaction of sodium valproate and parahydroxyphenobarbital. Addition of POHPB to the incubation mixture did not modify the enzyme activity towards VPA. For example, in one experiment, UDPGT activities were respectively 2.37 and 2.12 nmol/min/mg protein with and without POHPB.

To simulate chronic PB treatment in humans, glucuronidation of POHPB was studied in liver microsomes from rats pretreated with PB. Addition of VPA to the incubation mixture caused a competitive inhibition of the glucuronyltransferase. The apparent constant of inhibition K_i, calculated from Dixon plot, was 1.2×10^{-3} M (Fig. 2). Calculation of K_m of the enzyme with VPA or POHPB as substrate gave values of 0.79 and 1.15 mM respectively; values obtained from Lineweaver–Burk and Hofstee plots were very similar. Thus, the VPA affinity for the enzyme tended to be higher than that of POHPB.

Previous studies have shown that UDPGT activity towards POHPB is enhanced by PB pretreatment [23]. Thus, at least in rat liver microsomes, both VPA and POHPB might be glucuronidated by the same group of enzymes. Our results show that *in vitro* VPA conjugation is not affected by the presence of POHPB in the incubation mixture, but that POHPB conjugation is competitively inhibited by VPA. In order to tentatively explain the mechanism of this interaction one can postulate that these two molecules are glucuronidated at two different sites of the enzyme; the difference in the structure of these two molecules could allow VPA to compete with POHPB at its specific site. These *in vitro* data corroborate *in vivo* findings. In patients receiving both VPA and PB, a decrease in the glucuronidated fraction of POHPB but not in the total amount of POHPB has been reported [5]. Our study in rat microsomes is an approach which might explain the VPA–PB interaction. However, further investigations are needed to determine whether inhibition of PB hydroxylation, as shown by Kapetanovic [8], and of POHPB

Table 1. Influence of rat pretreatment on cytochrome P-450 and UDP glucuronyltransferase activities towards sodium valproate

Pretreatment	Cytochrome P-450 nmol/mg microsomal protein	UDP glucuronyltransferase nmol/min.mg microsomal protein
Control	0.57 ± 0.028	3.59 ± 0.16
Sodium valproate	0.62 ± 0.06	3.94 ± 0.16
Phenobarbital	1.14 ± 0.028*	5.05 ± 0.45†

Results are mean ± S.E.M. of at least three different experiments.

Rats were pretreated as follows: control (saline), phenobarbital 100 mg/kg/day, sodium valproate 200 mg/kg/day were administered i.p. daily for 3 days.

* Statistically different from control $P < 0.001$.

† Statistically different from control $P < 0.01$.

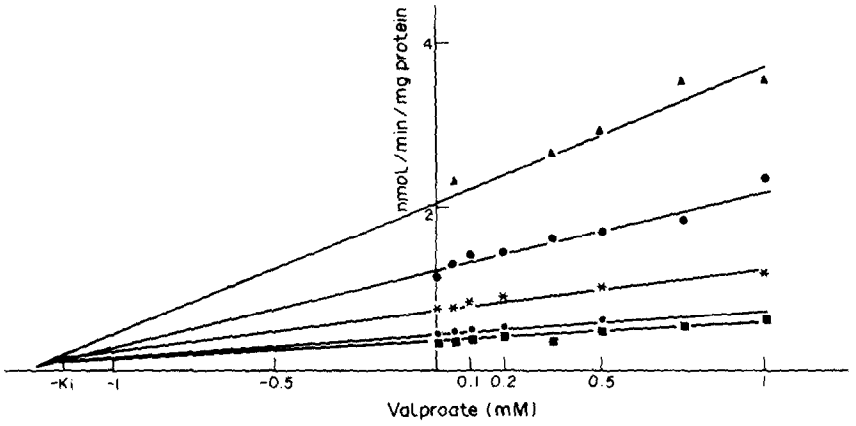

Fig. 2. Inhibition of the conjugation of parahydroxyphenobarbital by different concentrations of sodium valproate. Results are plotted according to Dixon. Concentrations of parahydroxyphenobarbital are: ▲, 0.05 mM; ●, 0.10 mM; *, 0.15 mM; •, 0.25 mM; ◆, 0.35 mM.

glucuronidation simultaneously occurs *in vivo*. Indeed, VPA may be a non-specific inhibitor of several drug metabolizing enzymes, as reported for other fatty acids [24–26].

In the present paper, the kinetics of UDP glucuronyltransferase towards valproate was studied in rat liver microsomes. The enzyme was activated by Triton X-100 and induced by phenobarbital whereas valproate pretreatment had no effect. POH PB, a PB metabolite did not inhibit valproate glucuronidation. Conversely valproate was found to competitively inhibit POH PB glucuronidation (K_i 1.2 mM) in rat liver microsomes. These results further characterize the valproate–phenobarbital interaction.

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