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STUDIES ON THE REACTIVITY OF ACYL GLUCURONIDES—III

GLUCURONIDE-DERIVED ADDUCTS OF VALPROIC ACID AND PLASMA PROTEIN AND ANTI-ADDUCT ANTIBODIES IN HUMANS*

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Abstract—The major metabolite of the anti-epileptic agent valproic acid (VPA) is its acyl glucuronide conjugate (VPA-G), which undergoes non-enzymic, pH-dependent rearrangement via acyl migration to a mixture of β-glucuronidase-resistant forms (collectively VPA-G-R). We have compared the reactivity of VPA-G and VPA-G-R towards covalent VPA–protein adduct formation by incubation in buffer, human serum albumin (HSA) and fresh human plasma at pH 7.4 and 37°. In all three media, the predominant reaction of VPA-G over 30 hr was rearrangement to VPA-G-R (*ca.* 24%). Hydrolysis was quite minor (*ca.* 2%) and covalent adduct formation negligible (when protein was present). On the other hand, both hydrolysis (*ca.* 27%) and adduct formation (*ca.* 7%) were extensive when VPA-G-R was incubated with HSA or plasma. These data do not support a transacylation mechanism for VPA–protein adduct formation, since this pathway should be much more highly favoured by VPA-G (an acyl-substituted acetal) than VPA-G-R (simple esters). VPA–protein adducts were found in the plasma of epileptic patients taking VPA chronically (mean 0.77 ± SD 0.63 μg VPA equivalents/mL, N = 17). An enzyme linked immunosorbent assay was developed, using HSA modified by incubation with VPA-G-R, to test the immunoreactivity of the patients' plasma. Of 57 patients tested, nine showed measurable levels of antibodies to these adducts, but the titres were very low, with no difference in response to modified and unmodified protein detectable at plasma dilutions of 1:16 or greater. These results suggest that the VPA–protein adducts have little immunogenicity, and are in agreement with clinical observations that drug hypersensitivity responses have not been associated with VPA therapy. Thus, although the *in vitro* data show that VPA-G is an example of a relatively unreactive acyl glucuronide, covalent VPA–plasma protein adducts and anti-adduct antibodies are nonetheless formed *in vivo*, at least in some patients on chronic therapy with the drug.

Drugs bearing carboxylic acid groups frequently form acyl glucuronides as major metabolites, and evidence has steadily accumulated over the past decade pointing to the potential reactivity of these conjugates, both *in vivo* and in biological samples *ex vivo* [1–3]. Three reactions—hydrolysis, rearrangement and covalent binding to protein—have been variously demonstrated, and these are related, directly or indirectly, to the intrinsic susceptibility to nucleophilic substitution reactions of the ester group linking the drug and glucuronic acid moieties. This reactivity of the ester group is the feature which distinguishes acyl glucuronides from other classes of glucuronide conjugates.

Hydrolysis of an acyl glucuronide conjugate leads to regeneration of the pharmacologically active parent drug: potential catalysts include hydroxide ion, β-glucuronidases, serum albumin and esterases [4–11], though the prime candidates *in vivo* seem to be esterases [12–14]. Rearrangement of acyl glucuronides (Fig. 1) occurs by non-enzymic intramolecular migration of the drug moiety between adjacent hydroxy groups on the glucuronic acid ring (Ref. 15 and references therein). The isomers thus formed are not glucuronides (a term reserved for the 1-β biosynthetic product) and are not hydrolysed by β-glucuronidases. The migrations are reversible, with the exception of reformation of the glucuronide itself. Finally, acyl glucuronides can undergo transacylation reactions with small chemical nucleophiles [16–18], and have been documented to form covalent adducts with plasma protein, notably serum albumin [8, 19–26]. The mechanism of the covalent binding reaction(s) has not been established, though both transacylation [8, 19] and glycation [20, 27] pathways have been proposed. The transacylation pathway involves nucleophilic transfer of the drug moiety from glucuronic acid to acceptor -SH, -OH or $-NH_2$ groups on protein, and should be highly favoured by the 1-β-glucuronide itself as compared to its 2-, 3- and 4-acyl migration isomers [18]. Conversely, the glycation or Schiff's base/Amadori pathway *requires* prior isomer formation to permit ring-opening of the glucuronic acid moiety: the aldehyde group so formed can then condense with $-NH_2$ groups of protein [20, 27]. Irrespective of the

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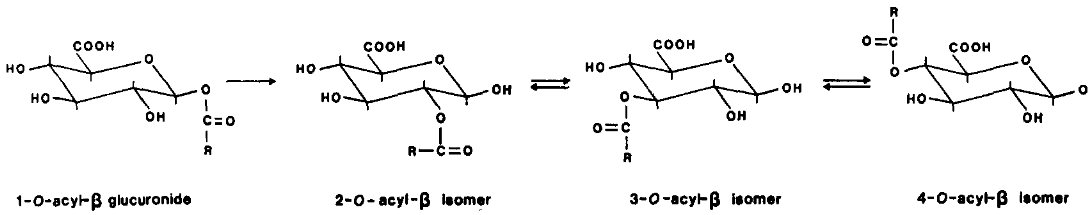

Fig. 1. Scheme showing rearrangement of the biosynthetic, β-glucuronidase-susceptible acyl glucuronide of a carboxylic drug (RCOOH) by acyl migration to the β-glucuronidase-resistant 2-, 3- and 4-*O*-acyl-β isomers.

operative mechanism, covalent attachment of drug or drug–glucuronic acid moieties to protein *in vivo* constitutes chemical modification of native protein, and has been suggested as a possible mediator of toxic responses [1, 3, 19, 20]. In particular, the drug–protein or drug–glucuronic acid–protein adducts could act as immunogens in the induction of drug hypersensitivity (the "hapten hypothesis") [28].

In some earlier work probing the reactivity of acyl glucuronides, we have studied the widely used anticonvulsant agent valproic acid (VPA*) as a model carboxylic drug. Direct glucuronidation is the major metabolic pathway in both rats [29] and humans [30]. The glucuronide conjugate (VPA-G) undergoes rearrangement to β-glucuronidase-resistant forms *in vitro* [6]: rearrangment was also documented in a patient with VPA-associated renal dysfunction [31]. In rat studies, comparison of the dispositions of VPA-G and a mixture of its β-glucuronidase-resistant rearrangement products (VPA-G-R) after separate administration revealed some major differences. For example, VPA-G-R was much more resistant to hydrolysis in the gut after intrabiliary administration (thereby substantially reducing enterohepatic recirculation [32]) and systematically after i.v. administration (thereby contributing to its apparent impaired biliary excretion [33]). The present study was carried out to compare hydrolysis and protein adduct formation from VPA-G and VPA-G-R in plasma and serum albumin solutions *in vitro*, to determine whether VPA–plasma protein adducts were formed in patients on chronic therapy with this anticonvulsant, and if so, to ascertain whether antibodies to these adducts were present in the patients' plasma.

MATERIALS AND METHODS

Materials. VPA, nonanoic acid, casein, sodium azide, β-glucuronidase (EC 3.2.1.31, from *Helix pomatia*, type H-2), sodium heparin (from porcine intestinal mucosa) and HSA (Product A8763, essentially globulin- and fatty-acid free: 0.5% Alpha 1 globulin, 0.4% Alpha 2 globulin and <0.005% fatty acids) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Solvents (HPLC grade) and reagents (analytical grade) were obtained from commercial sources.

Preparation of VPA-G and VPA-G-R. VPA-G was biosynthesized by administration of VPA to rats, and VPA-G-R prepared from VPA-G by intramolecular rearrangement, as described previously [32]. In brief, male Sprague–Dawley-derived rats (300–350 g) were obtained from The University of Queensland Medical Faculty Animal House, and fitted (under ether anaesthesia) with catheters in the jugular vein and bile duct (experiments were approved by The University of Queensland Animal Experimentation Ethics Committee). Following recovery from anaesthesia, the rats were dosed each 2 hr with 150 mg VPA/kg i.v. (for up to four doses), and the bile (containing *ca.* 60% of the dose as VPA-G [29]) collected over dry ice.

Partial purification of VPA-G was effected by adjustment of the pH to 2 with 2 M HCl, centrifugation, extraction of the supernatant three times with 3 vol. of 1-chlorobutane to remove VPA, followed by extraction three times with 3 vol. of diethyl ether. The ether extracts were combined and evaporated to dryness under a stream of nitrogen gas. The pale syrupy residue was taken up in water and the pH carefully adjusted to 5 with 0.5 M NaOH. The solution was made up to contain VPA-G at *ca.* 6 mg VPA equivalents/mL, and was contaminated by ≤0.1% VPA and *ca.* 1.2% VPA-G-R. For preparation of VPA-G-R by pH-dependent intramolecular rearrangement, the pH of bile containing VPA-G was adjusted to 9.2 with 2 M NaOH, and the solution incubated at 37° for 34 hr. The pH was then adjusted to 5 with 2 M HCl, 2000 units β-glucuronidase/mL bile were added, and the mixture incubated at 37° for 3 hr to hydrolyse remaining VPA-G. The pH was then adjusted to 2 with 2 M HCl, and the same procedure described above for extraction of VPA-G followed. The final solution was made up to contain VPA-G-R at *ca.* 6 mg VPA equivalents/mL, and was contaminated by ≤0.2% VPA and *ca.* 2.0% VPA-G.

Incubation of VPA-G and VPA-G-R with blood components and tissue homogenates. Under ether anaesthesia, rats were heparinized (*ca.* 170 U sodium heparin in 0.1 mL saline) and exsanguinated via the aorta. Blood samples (10 mL) were then transferred to tubes containing 125 U heparin. Plasma was

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* Abbreviations: VPA, valproic acid; VPA-G, valproic acid glucuronide; VPA-G-R, acyl migration rearrangement products of valproic acid glucuronide; HSA, human serum albumin; ELISA, enzyme linked immunosorbent assay; TBS, Tris-buffered saline; $T_{1/2}$, half-life.

separated after centrifugation. The packed red blood cells were washed three times by resuspension in saline, centrifugation and removal of the washings and residual white cell layer, and reconstituted to original volume with saline. Lysed red blood cells were obtained by reconstituting the washed cells to original volume in water, freezing (dry ice) and thawing. The rat liver, kidneys, and caecum and contents were homogenized (Ika Ultraturrax, Janke and Kunkel, Staufen, F.R.G.) in 5 vol. of 0.1 M sodium phosphate buffer pH 6.8. Components of human blood were obtained by the same procedures used for rat blood.

For the incubation experiments, whole blood, plasma or lysed red cells (3 mL samples) and 1 M sodium phosphate buffer (pH 7.1, 0.3 mL) were incubated at 37° for 3 hr with 0.1 mL of the VPA-G or VPA-G-R stock solutions (i.e. *ca.* 600 μg VPA equivalents). Samples (0.5 mL) were removed at 0.25, 1, 2 and 3 hr, mixed with 0.5 M HCl (1 mL), and analysed for liberated VPA. The rat tissue homogenates (6 mL) were incubated at 37° for 3 hr with 1 mL of the VPA-G or VPA-G-R stock solutions (i.e. 6000 μg VPA equivalents). Samples (0.5 mL) were removed at 0.25, 0.5, 0.75 and 1 hr (caecum and contents) or 0.25, 1, 2 and 3 hr (liver or kidney), mixed with 0.5 M HCl (1 mL), and analysed for liberated VPA. Hydrolysis rates were calculated on a per mL fluid or per g tissue basis.

Incubation of VPA-G and VPA-G-R in buffer, HSA solutions and human plasma. VPA-G and VPA-G-R were incubated at 300 μg VPA equivalents/mL at 37° for 30 hr in solutions of phosphate buffer (0.1 M, pH 7.4), HSA (38 mg/mL in 0.1 M phosphate buffer, pH 7.4) and fresh human plasma buffered to pH 7.4 (4.5 mL plasma, 0.45 mL 1 M phosphate buffer, pH 7.1). The solutions were prewarmed to 37° and incubations initiated by the addition of stock VPA-G or VPA-G-R. Three samples ($2 \times 100\,\mu$L and $1 \times 500\,\mu$L) of each incubation mixture were taken immediately and at 1, 4, 8, 24 and 30 hr for analysis of VPA, VPA-G and VPA-G-R (100 μL samples) and of covalently bonded VPA (500 μL sample).

A longer term incubation of HSA with VPA-G and VPA-G-R using the same concentrations and conditions was undertaken in the presence of 0.05% (w/v) sodium azide to inhibit bacterial growth. Samples were taken as above and at 2, 8, 15, 22, 29, 36, 43 and 50 days.

Plasma samples from patients. Plasma samples from patients on chronic VPA therapy were obtained from the Royal Brisbane Hospital Department of Pathology after routine therapeutic drug monitoring of VPA and other anticonvulsants, and frozen until analysis. The patients studied comprised 32 females and 25 males, ranging in age from 3 to 75 years (mean 31 ± SD 17 years). Only six subjects were younger than 14 years. Sodium VPA doses ranged from 400 to 4000 mg/day (mean 1450 ± SD 790 mg/day). Thirty-seven of the 57 subjects received other anticonvulsants (carbamazepine in 22, phenytoin in 12, phenobarbital or methylphenobarbital in 10, clonazepam in four and ethosuximide in two subjects). Non-conjugated VPA, albumin and total protein concentrations in the plasma samples were measured by the Department of Pathology using automated analysis techniques.

Analysis of VPA, VPA-G and VPA-G-R. All analyses were performed by measurement of VPA itself (after β-glucuronidase or alkaline hydrolysis if appropriate), using our previously published gas chromatographic procedure which employs nonanoic acid as internal standard [6]. In brief, non-conjugated VPA was measured directly after extraction of acidified samples with 1-chlorobutane (which does not extract VPA-G or VPA-G-R). Total "conjugated" VPA (i.e. VPA-G and VPA-G-R) was then obtained from the residues following washing and alkaline hydrolysis. VPA from VPA + VPA-G was measured following incubation of the second 100 μL sample with β-glucuronidase at pH 5 and extraction with 1-chlorobutane. Alkaline hydrolysis of the washed aqueous residues then provided a direct measurement of VPA from VPA-G-R. From these two procedures VPA and VPA-G-R were obtained directly and VPA-G (two estimations) by subtraction methods.

Analysis of covalently bonded VPA. For analysis of covalently bonded VPA in the 500 μL samples, 1.5 mL of 4% (v/v) acetic acid in acetonitrile were immediately added, and the resultant suspension sonicated for 10 min, and then centrifuged. The supernatant was discarded and the protein pellet resuspended in 1.5 mL of a 3:1 (v/v) mixture of 4% acetic acid in acetonitrile and water, vigorously vortexed and centrifuged. This washing procedure was repeated nine times. The pellet was dried under a gentle stream of air and digested in 1 M NaOH (500 μL) at 65° overnight. After cooling, the digest was acidified with 10 M HCl (100 μL), and 100 μL of internal standard solution (1 mg nonanoic acid per mL chloroform) added. The mixture was equilibrated and centrifuged, and a 1–2 μL sample of the chloroform layer injected into the gas chromatograph. Standard curves were constructed using washed, blank plasma or HSA protein pellets spiked with 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10 μg VPA. The spiked pellets were base-digested and extracted as for samples. Standard curves were linear with correlation coefficients exceeding 0.999. The minimum quantifiable concentration was set at 0.1 μg VPA/pellet (0.2 μg VPA/mL). Appropriate control experiments confirmed the adequacy of the washing procedure for complete removal of non-covalently bonded VPA from the protein pellet.

For analysis of covalently bonded VPA in 0.5 mL patient plasma samples, increased sensitivity (minimum quantifiable concentration 0.1 μg VPA/mL) was achieved by use of a BP21 megabore capillary column (SGE, Melbourne, Australia) instead of the packed column. Injector and detector temperatures were 190° and 200°, respectively. The carrier gas was helium at a flow rate of 7 mL/min with nitrogen as detector make-up gas. The oven was programmed from 120° (4 min hold) to 165° (1 min hold) at 5°/min.

Preparation of VPA-modified protein for antibody measurement. VPA-modified protein was obtained by incubating VPA-G-R (1.6 mg VPA equivalents/mL) with HSA (32 mg/mL) in 0.1 M phosphate buffer, pH 7.4 at 37° for 28 hr. A 500 μL sample was taken to determine the extent of adduct formation and the remainder frozen until required. A control sample of "unmodified" HSA was prepared by parallel incubation of HSA with an extract obtained from blank rat bile in the same way as VPA-G-R had been obtained from the bile of rats dosed with VPA.

Measurement of antibody binding to VPA-modified protein. An ELISA was used to measure antibody levels in plasma samples. The ELISA was based on that described by Worrall *et al.* [34], and utilized the Amersham biotin/streptavidin system (Amersham International, Amersham, U.K.). Microtitre plates (AS Nunc, Roskilde, Denmark) were coated with 100 μL of unmodified HSA or VPA-modified HSA solutions (50 μg/mL in 0.1 M sodium carbonate/bicarbonate buffer, pH 9.6) overnight at 4°. The plates were washed with TBS, pH 7.4 containing 0.5% (w/v) casein (TBS–casein), and non-specific binding sites blocked by incubation with saturated casein solution for 1 hr at room temperature. The patient plasma samples were serially diluted in TBS–casein, with each dilution (100 μL) being added to the wells. The plates were then incubated for 1 hr at 37°. After washing with TBS–casein, 100 μL of biotinylated goat anti-human immunoglobulin (not class-specific) (diluted 1:5000 in TBS–casein) were added to each well and incubated for 1 hr at 37°. The plates were again washed with TBS–casein and 100 μL of streptavidin/alkaline phosphatase complex (diluted 1:1000 in TBS–casein) were added. After 30 min incubation at 37°, the wells were washed as before and 100 μL of *p*-nitrophenyl phosphate (1 mg/ml) in diethanolamine buffer (10 mM diethanolamine, 0.5 mM $MgCl_2$, pH 9.6) were added. After 20 min incubation at 37°, the absorbance for each well was read at 405 nm using a Titertek Multiskan plate reader. Antibody binding to VPA-modified HSA was defined as the difference in absorbance between wells coated with unmodified and modified HSA.

RESULTS

Initial rates of hydrolysis of VPA-G and a mixture of its β-glucuronidase-resistant rearrangement products, VPA-G-R, in fresh human and rat blood and some rat tissues are shown in Table 1. Hydrolysis was more rapid in all biological samples than in buffer at the same pH. The rat tissue homogenates (particularly caecum and contents) showed very high hydrolytic activity towards VPA-G, exceeding that towards VPA-G-R by about an order of magnitude. Hydrolysis of VPA-G in rat whole blood and lysed red cells was also extensive and considerably greater than that found for VPA-G-R. By contrast, hydrolysis of both VPA-G and VPA-G-R in rat plasma was quite minor. In fractions of fresh human blood, extensive hydrolysis was observed only in lysed red cells. In both human whole blood and plasma, rates of hydrolysis of VPA-G were significantly [$P < 0.01$ (Student's *t*-test)] lower than those of VPA-G-R.

The profiles of rearrangement, hydrolysis and adduct formation following incubation of VPA-G and VPA-G-R (initial concentrations *ca.* 300 μg VPA equivalents/mL, i.e. 2.08 mM) in buffer, HSA and human plasma solutions for 30 hr are shown in Figs 2–4. In pH 7.4 buffer, rearrangement of VPA-G into β-glucuronidase-resistant VPA-G-R was the only appreciable reaction (24%, Fig. 2). Hydrolysis accounted for only 2% of either VPA-G or VPA-G-R substrate after 30 hr incubation. The profiles of rearrangement and hydrolysis of VPA-G were little altered by incubation in either HSA solution (17% and 1.6%, respectively, Fig. 3) or in fresh human plasma (29% and 3.8%, respectively, Fig. 4), and negligible covalent binding of VPA to the protein occurred (≤0.1% of substrate VPA-G was recovered as VPA–protein adducts i.e. ≤5 ng VPA/mg protein). By contrast, hydrolysis of VPA-G-R was greatly accelerated (*ca.* 13-fold) in either HSA solution or plasma (Figs 3 and 4), and formation of VPA–protein adduct was extensive. Thus, 6.4 and 7.7% of substrate VPA-G-R were recovered as VPA–protein adducts after 30 hr incubation in the HSA and plasma solutions, respectively (i.e. 540 and 370 ng VPA/mg protein, respectively). In this context it should be noted that the pre-formed VPA-G-R mixture used in the incubations was a better substrate for adduct formation than the VPA-G-R generated from VPA-G *in situ* e.g. 17 and 29% of substrate VPA-G had rearranged to VPA-G-R during 30 hr incubation in HSA solution and plasma, respectively, but concurrent adduct formation was negligible. Appropriate control incubations verified that incubation of HSA with VPA itself failed to yield detectable VPA–protein adducts.

Since VPA–protein adduct formation via VPA-G-R had not reached a maximum and no appreciable binding via VPA-G had been obtained by 30 hr, long term incubations of the substrates with HSA were undertaken in the presence of 0.05% (w/v) sodium azide (to inhibit bacterial growth). VPA-G concentration declined in a log–linear manner ($T_{1/2}$ = 4.5 days, Fig. 5), yielding primarily VPA-G-R which peaked in concentration at 8 days (at 53% of the initial VPA-G concentration) and declined in turn with a $T_{1/2(15\text{–}50\,\text{days})}$ of 9.9 days. Appreciable adduct formation was not observed until 8 days. By contrast, adduct formation mediated by incubation of HSA with preformed VPA-G-R was rapid and extensive (Fig. 6). The adduct concentrations peaked at 2 days (610 ng VPA/mg protein, i.e. 1 mol VPA/3.6 mol HSA) and then declined roughly in parallel with substrate VPA-G-R ($T_{1/2(8\text{–}50\,\text{days})}$ = 9.9 days), at least until about 30 days. To establish both the essential integrity of the protein during long term incubation and the absence of effect of sodium azide on adduct formation, a control incubation of HSA at 37° (in the presence of sodium azide) was carried out for 10 days prior to addition of VPA-G-R. The 30 hr hydrolysis and covalent binding profiles obtained were very similar to those obtained in the absence of the bacteriostat (Fig. 3).

Plasma samples from patients on chronic VPA therapy were obtained after routine anticonvulsant drug monitoring to determine whether VPA–protein adducts were formed *in vivo*. The mean plasma VPA level found in 57 patient plasma samples was 47 ± SD 22 μg VPA/mL (range 10–112 μg/mL). VPA–protein adducts were detectable in all of 22 patient samples whose volumes permitted this

Table 1. Hydrolysis of valproic acid glucuronide (VPA-G) and its acyl migration rearrangement forms (VPA-G-R) at 37°

	μg VPA released/hr per mL fluid or per g tissue	
	VPA-G substrate	VPA-G-R substrate
Human		
Whole blood* (N = 7)	3.8 ± 0.27	5.2 ± 0.10
Plasma* (N = 6)	1.4 ± 0.13	3.9 ± 0.09
Lysed red blood cells* (N = 4)	97 ± 2.9	30 ± 1.4
Rat		
Whole blood* (N = 5)	64 ± 7.6	12 ± 1.3
Plasma* (N = 4)	1.0 ± 0.38	1.3 ± 0.33
Lysed red blood cells* (N = 3)	110 ± 22	48 ± 2.2
Homogenized liver† (N = 3)	980 ± 34	57 ± 3.4
Homogenized kidney† (N = 3)	330 ± 17	44 ± 0.6
Homogenized caecum and contents† (N = 3)	4600 ± 1200	140 ± 41
Buffer		
0.1 M phosphate pH 7.4	0.20	0.18

Results are means ± SE, using blood/tissues from N volunteers or rats.

* Buffered to *ca.* pH 7.4.

† Homogenized in 5 vol. of 0.1 M phosphate buffer pH 6.8.

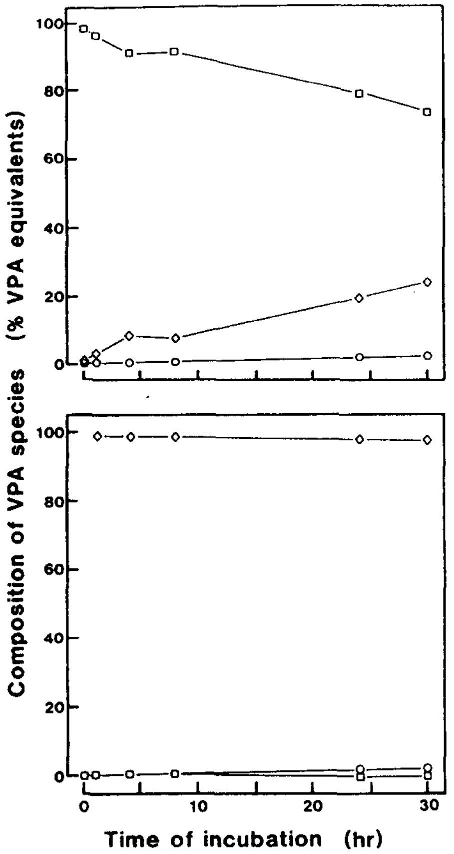

Fig. 2. Profiles for rearrangement of valproic acid glucuronide (VPA-G, □) to its *β*-glucuronidase-resistant forms (VPA-G-R, ◊) and for hydrolysis to valproic acid (VPA, ○) following incubation of VPA-G (upper panel) and VPA-G-R (lower panel) at initial concentrations of *ca.* 300 μg VPA equivalents/mL in 0.1 M phosphate buffer pH 7.4 at 37°.

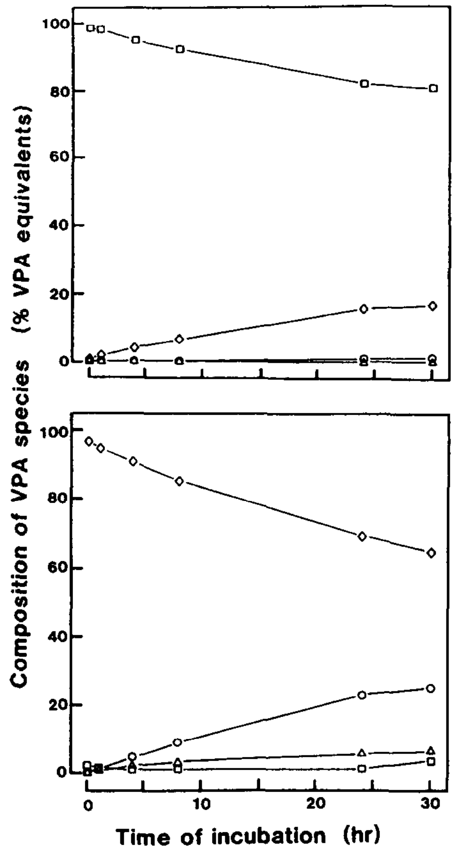

Fig. 3. Profiles for rearrangement of valproic acid glucuronide (VPA-G, □) to its *β*-glucuronidase-resistant forms (VPA-G-R, ◊), for hydrolysis to valproic acid (VPA, ○) and for covalent binding to protein (VPA–HSA adduct, △) following incubation of VPA-G (upper panel) and VPA-G-R (lower panel) at initial concentrations of *ca.* 300 μg VPA equivalents/mL at 37° in 0.1 M phosphate buffer pH 7.4 containing HSA at 38 mg/mL.

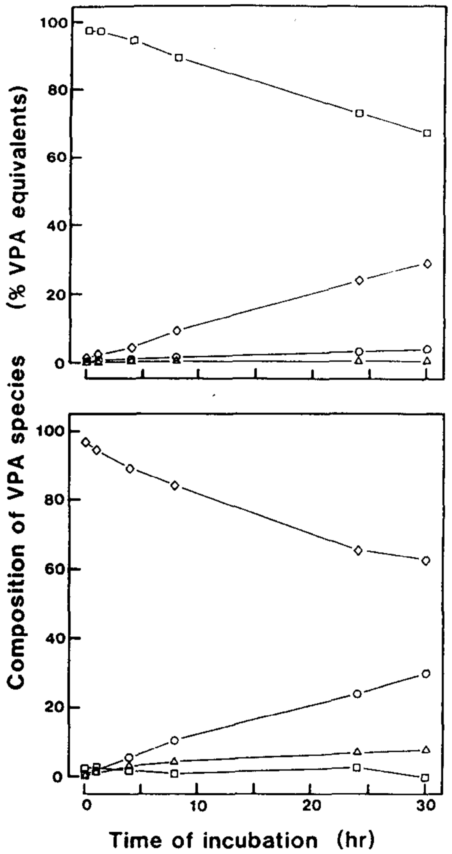

Fig. 4. Profiles for rearrangement of valproic acid glucuronide (VPA-G, □) to its β-glucuronidase-resistant forms (VPA-G-R, ◊), for hydrolysis to valproic acid (VPA, ○) and for covalent binding to protein (VPA–protein adduct, △) following incubation of VPA-G (upper panel) and VPA-G-R (lower panel) at initial concentrations of *ca.* 300 μg VPA equivalents/mL at 37° in fresh human plasma buffered to pH 7.4.

analysis, but measurable only in 17 samples (mean 0.77 ± SD 0.63 μg VPA equivalents/mL, range 0.22–2.38 μg/mL). The mean value of 0.77 μg VPA equivalents/mL plasma corresponds approximately to 11 ng VPA covalently bound/mg protein (based on 70 mg protein/mL plasma) or to 1 mol VPA covalently bound/130 mol HSA (based on 45 mg HSA/mL plasma) *if* HSA were the only plasma protein involved in the reaction. In 11 of these 17 samples, sufficient volume also permitted concentrations of total "conjugated" VPA (i.e. VPA-G + VPA-G-R) to be determined (mean 1.9 ± SD 3.2 μg VPA equivalents/mL, range 0.62–11.4 μg/mL). Data from the 11 patient samples are shown in Table 2. Sample volume limitations precluded separate estimation of VPA-G and VPA-G-R.

To test the immunoreactivity of the patient plasma samples with VPA–protein adducts, an ELISA procedure was developed using HSA modified by incubation with VPA-G-R (i.e. VPA–HSA adduct at 1100 ng VPA equivalents/mg protein, Materials and Methods). As the VPA-G-R used had been obtained by extraction of rearranged VPA-G originally excreted in the bile of rats dosed with VPA (Materials and Methods), "unmodified" HSA for the control ELISA assay was prepared by similar incubation of HSA with an equivalent extract obtained from blank rat bile. Eighteen of the 57 patient plasma samples were assayed by the ELISA procedure using serial dilutions of plasma. Three responded positively i.e. had elevated immunoreactivity with the VPA-modified protein. However, the antibody titres were very low, with no difference in response to modified and unmodified HSA being detectable at plasma dilutions of 1:16 or greater (Fig. 7). As the difference in response was greatest at plasma dilutions between 2- and 8-fold, the remaining 39 samples were screened in duplicate at one dilution (4-fold). This revealed six positives, as defined by a difference in absorbance (between wells coated with modified and unmodified HSA) of greater than 0.2 U. Of the total of nine positives, VPA–protein adduct concentrations had been measured in three. Two of these were amongst the 11 patient samples detailed in Table 2. In the third, plasma concentrations of reversibly and covalently bound VPA were 16 and 2.4 μg VPA/mL, respectively.

DISCUSSION

Compared to the acyl glucuronide conjugates of most drugs examined, VPA-G rearranges to β-glucuronidase-resistant forms (VPA-G-R) quite slowly at physiological pH *in vitro* [6, 35] (see Figs 2–4). Thus, relatively little VPA-G-R would be expected to be formed *in vivo* during circulation prior to urinary excretion. This is supported by earlier work [31], which showed that (a) plasma concentrations of total "conjugated" VPA, but not of VPA-G or VPA-G-R individually, were measurable (at *ca.* 3% of concurrent VPA concentrations) in patients with normal renal function, and (b) VPA-G-R constituted only about 4.4% of the total "conjugated" VPA recovered in urine. The same study however found that systemic rearrangement of VPA-G was considerably enhanced (by about 5–10 fold) when its excretion was retarded in a patient with drug-associated renal dysfunction. These data suggest that rearrangement of VPA-G to VPA-G-R does occur systemically, albeit at a quantitatively minor level, in normal patients on VPA therapy. It was not possible to confirm this in the present study.

The *in vivo* disposition of VPA-G and VPA-G-R cannot be studied by their direct administration to humans. However, earlier *in vivo* work in rats found VPA-G to be hydrolysed much more readily than VPA-G-R in the gut after intrabiliary [32], or systemically after *i.v.* [33] administration of the preformed "conjugates". The present *in vitro* results (Table 1) confirm the capacity of rat tissues such as gut, liver and kidney (at least as homogenates) to hydrolyse VPA-G rapidly. By comparison, the contribution of the blood compartment itself to hydrolysis of either VPA-G or VPA-G-R was quite modest, and largely confined to the red cell

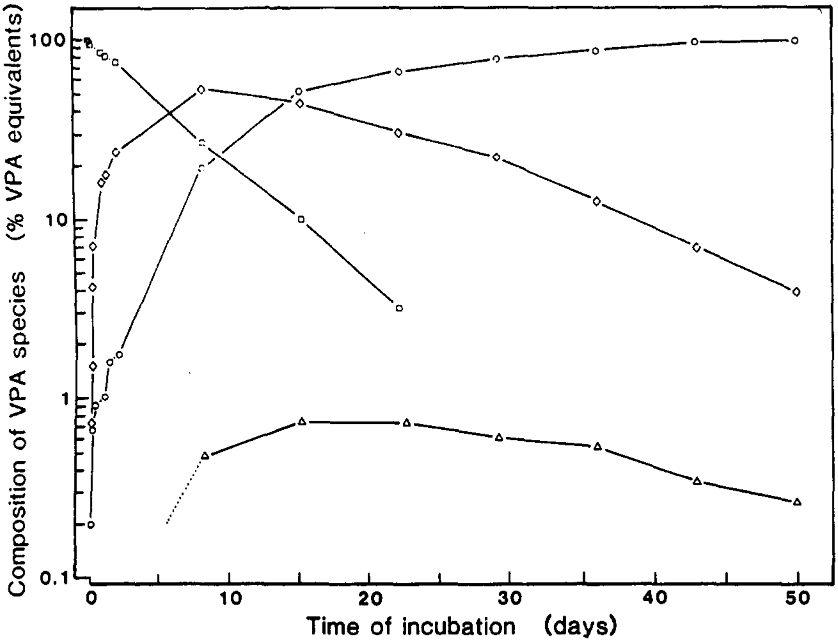

Fig. 5. Profiles for rearrangement of valproic acid glucuronide (VPA-G, □) to its β-glucuronidase-resistant forms (VPA-G-R, ◊), for hydrolysis to valproic acid (VPA ○) and for covalent binding to protein (VPA–HSA adduct, △) following long term incubation of VPA-G at an initial concentrations of *ca.* 300 μg VPA equivalents/mL at 37° in 0.1 M phosphate buffer pH 7.4 containing HSA (38 mg/mL) and 0.05% (w/v) sodium azide.

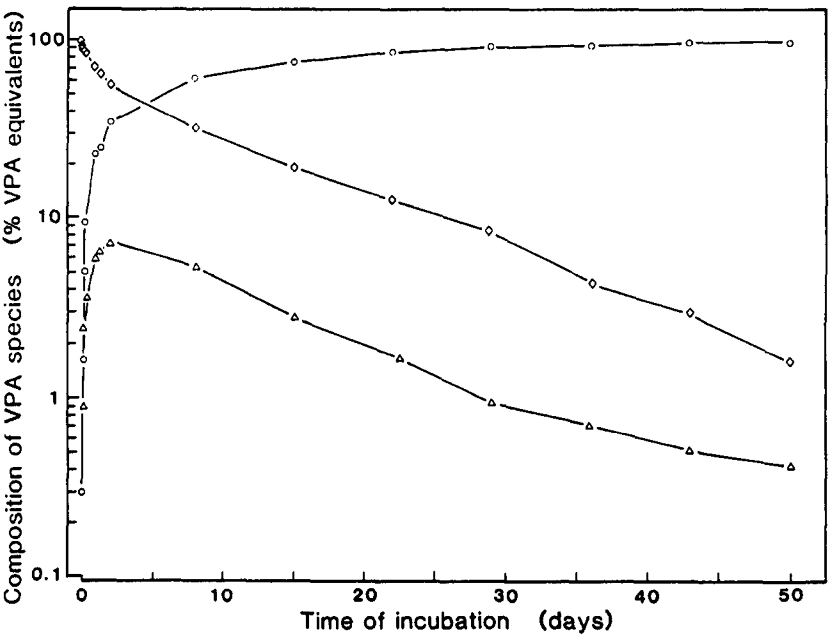

Fig. 6. Profiles for hydrolysis of β-glucuronidase-resistant forms of valproic acid glucuronide (VPA-G-R, ◊) to valproic acid (VPA ○) and for covalent binding to protein (VPA–HSA adduct, △) following incubation of VPA-G-R at an initial concentrations of *ca.* 300 μg VPA equivalents/mL at 37° in 0.1 M phosphate buffer pH 7.4 containing HSA (38 mg/mL) and 0.05% (w/v) sodium azide.

Table 2. Some patient details and plasma concentrations of VPA species in 11 of 57 patient plasma samples tested for antibodies to VPA–HSA adducts

				Plasma concentration (μg VPA equivalents/mL)			
Subject	Age	Sex	VPA dose (mg/day)	VPA	VPA-G + VPA-G-R	VPA–protein adduct	Antibody detected*
1	42	M	NA	30	0.76	0.35	–
2	46	F	600	38	0.90	0.73	–
7	58	F	1700	29	1.21	0.47	–
8	45	M	NA	67	11.40	1.89	–
9	35	F	800	31	0.84	0.36	–
13	44	F	NA	54	1.25	0.75	–
17	37	F	2100	34	1.19	0.40	–
22	19	M	800	30	0.62	0.22	+
24	31	F	NA	57	0.90	0.39	+
25	34	F	NA	49	1.04	0.53	–
53	31	M	4000	112	1.20	1.60	–

NA, daily dose data not available.

* Positive result taken as an absorbance difference ≥ 0.2 in the ELISA at a 4-fold plasma dilution.

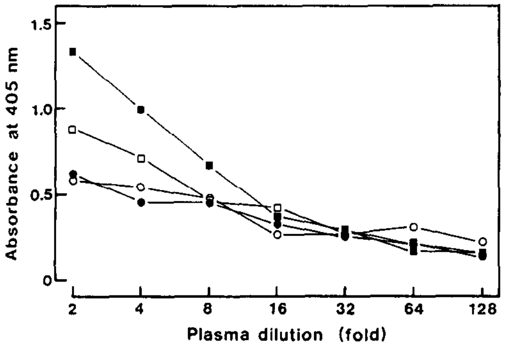

Fig. 7 Response in the ELISA assay of serially diluted plasma samples from two patients on chronic VPA therapy to VPA-modified (■, ●) and unmodified (□, ○) HSA. The profiles are representative of positive (■, □) and negative (●, ○) responses.

component (Table 1). Rat plasma had little hydrolytic capacity towards VPA-G, with similar results for VPA-G-R. As relatively polar chemical entities, VPA-G and VPA-G-R would not be expected to be rapidly taken up into red blood cells, but the rates of hydrolysis in whole blood versus plasma give cause to question such an assumption, at least for rat blood. In human whole blood and plasma, VPA-G was, surprisingly, hydrolysed more slowly than VPA-G-R, though both rates were modest in comparison to those in lysed red cells. These data point not only to species differences in hydrolysis rates in the blood compartment but also to catalysts other than β-glucuronidases and hydroxide ion.

The greater reactivity of VPA-G-R compared to its precursor VPA-G towards hydrolysis in human plasma was supported by the 30 hr incubation experiments (Figs 2–4), in which covalent binding of VPA to protein was examined as well. Thus, the predominant reaction of VPA-G in buffer, HSA and human plasma was rearrangement to VPA-G-R, with hydrolysis being quite minor, though greater than covalent binding when protein was present. By contrast, degradation of VPA-G-R by hydrolysis in plasma or HSA solutions was greatly enhanced compared to the rate in buffer alone. These data suggest a catalytic role for HSA itself in hydrolysis of VPA-G-R, but not VPA-G. Similarly, VPA–protein adduct formation was much more rapid and extensive from VPA-G-R than from VPA-G (Figs 3 and 4). These results showing the greater reactivity of VPA-G-R should be interpreted in the light of two factors: (a) negligible covalent binding via VPA-G had occurred over 30 hr incubation, even though *ca.* one quarter of the VPA-G had rearranged to VPA-G-R *in situ* during this time, and (b) "VPA-G-R" refers, collectively, to a mixture (of unknown composition) of β-glucuronidase-resistant rearrangement products of VPA-G, arising from acyl migration (Fig. 1). Taken together, these points suggest that the individual components of VPA-G-R giving greatest hydrolysis and covalent binding to protein are distal rather than proximal to VPA-G in the rearrangement scheme. This result is amplified in the long term incubation studies (Figs 5 and 6) where appreciable adduct formation via VPA-G was not observed until *ca.* 8 days, when VPA-G-R concentration was almost double that of parent VPA-G. From these results it seems likely therefore that VPA-G itself does not yield covalent adducts with protein. Identification of the proportion of individual acyl migration isomers present in VPA-G-R samples has not been achieved (because of limitations in the assay methodology), and it is further conceivable [6, 32] that β-glucuronidase-resistant products in addition to the 2-, 3- and 4-*O*-positional isomers (as β- and α-anomers [18]) may contribute to "VPA-G-R".

Reports in the literature on the effects of albumin or plasma on the reactivity of acyl glucuronide conjugates and their isomers vary with the drugs studied. In the presence of HSA, degradation of the acyl glucuronides of diflunisal [24] and tolmetin [25] was retarded, whilst rearrangement of probenecid glucuronide was reduced but its hydrolysis and that of its isomers were increased [26]. Similar rates of rearrangement and hydrolysis of fenofibric acid glucuronide were reported in HSA and buffer solutions [22], although more rapid degradation in unbuffered plasma was observed. Degradation of zomepirac glucuronide in blood and plasma was found to be faster than in buffer [7]. Ruelius *et al.* [8] found that hydrolysis and rearrangement of oxaprozin glucuronide were accelerated in HSA and plasma, and that the 2-isomer was hydrolysed at about the same rate as the glucuronide itself. They further reported that covalent binding to protein occurred to a significant extent with oxaprozin glucuronide but not its 2-isomer, and presented evidence showing that the glucuronic acid moiety was lost during the covalent binding reaction. In other studies comparing covalent adduct formation via acyl glucuronides and their rearrangement isomers, tolmetin glucuronide [25] formed adducts with HSA more rapidly and to a greater extent that did its 3-isomer. Similarly, adduct formation from zomepirac glucuronide [20] was greater than from an unpurified mixture of its positional isomers. Clearly, the reactivities of acyl glucuronides and their isomers are strongly influenced by the structure of the drug moiety.

Although covalent binding of drugs to protein via their acyl glucuronide conjugates is well documented, the mechanism(s) of covalent attachment, the nature of the binding site(s) and the structures of the adducts are unknown. The two mechanisms proposed—direct transacylation, with loss of the glucuronic acid moiety [8, 19, 36, 37] and isomerization/glycation, with retention of the glucuronic acid moiety [20, 27]—should be distinguishable (*in vitro*) on the basis of which of the acyl glucuronide itself and its rearrangement isomers is the better reactant, given that the acyl migration rearrangement scheme (Fig. 1) does not include reformation of the glucuronide from its isomers. Thus, the present results with VPA-G and VPA-G-R do not favour the transacylation pathway, and provide indirect support for the isomerization/glycation mechanism. The interpretation is somewhat compromised, however, by our lack of knowledge of the exact composition of "VPA-G-R". Nonetheless, the net result is the same as that obtained in earlier work on diflunisal acyl glucuronide [18], where the purified individual isomers, particularly the 4-isomer, were much better substrates for adduct formation with HSA *in vitro* than the acyl glucuronide itself.

The biological significance of the covalent binding reaction is unknown, but it is possible that such modified proteins may be immunogenic and mediate toxic responses *in vivo* [19, 20]. Although it generally is believed that the molecular weight of a substance is the first characteristic that determines if it will induce an immune response, low molecular weight chemicals, such as drugs, may become immunogenic when linked to a macromolecule such as a protein. This ***hapten hypothesis*** is derived from classical experiments of Landsteiner and Jacobs [38], Gell *et al.* [39] and Eisen *et al.* [40]. The immunogenicity of drug–protein adducts is affected by many factors, including nature of the carrier macromolecule, degree of drug incorporation (epitope density), site of bioactivation and degree of antigenic foreignness [41, 42]. Covalent drug–protein adducts, presumably glucuronide-derived, have been detected in the plasma of human volunteers given single oral doses of zomepirac [20], tolmetin [21] and diflunisal [24] and short courses of diflunisal and probenecid [23]. On the other hand, detectable concentrations of the glucuronides of oxaprozin [8] and fenofibric acid [22] have not been found in plasma and these studies concluded that there could be little or no clinical significance for their *in vitro* results demonstrating the capacity of the glucuronides to yield drug–protein adducts.

VPA-G is detectable in the plasma of patients on VPA therapy, and has the capacity to rearrange to its isomers *in vivo* [31]. In the present study, patient plasma samples were found to contain VPA–protein adducts (Table 2), albeit at generally low levels (usually <2% of plasma VPA). Total conjugated VPA levels (i.e. VPA-G + VPA-G-R) correlated reasonably well with adduct concentrations ($r = 0.75$). Nonetheless, the presence of these adducts *in vivo*, presumably formed *via* the sequence VPA → VPA-G → VPA-G-R → adducts, is perhaps a little surprising, given the slow rearrangement of VPA-G and subsequent adduct formation observed *in vitro* ($T_{1/2}$ of VPA-G in HSA solution at pH 7.4 and 37° was 4.5 days, Fig. 5). Thus, the presence of the adducts in plasma of patients on chronic VPA therapy presumably reflects their very limited formation and relative stability. The terminal $T_{1/2}$ of the adducts in HSA solution *in vitro* was ≥10 days (Fig. 6). Similar data on adduct degradation *in vivo* cannot be obtained without major interruptions to VPA therapy. However, in volunteers given short courses of diflunisal and probenecid [23], plasma protein adducts of these drugs were eliminated with terminal $T_{1/2}$ values of 10 and 13.5 days, respectively.

It is of course possible that the VPA–protein adducts found in patients' plasma derive from a mechanism other than (or in addition to) acyl glucuronide reactivity. Porubek *et al.* [43] described the covalent attachment of low levels of radiolabel to intracellular protein of liver and some other tissues following administration of single 60 mg/kg doses of [2-^{14}C]VPA and its metabolite [2-^{14}C]Δ^4-VPA to rats. Incorporation of radioactivity into protein also occurred when the substrates were incubated with viable, but not heat-inactivated, rat hepatocytes. In these experiments, the kinetics of incorporation (an initial rapid phase followed by a slow phase) suggested at least two underlying mechanisms. They proposed one of these to involve cytochrome P450-catalysed desaturation of VPA to Δ^4-VPA followed by formation of the coenzyme A thioester and conversion by the fatty acid β-oxidation pathway to a reactive species, 3-keto-Δ^4-VPA, which could then bind covalently with sulphydryl groups of proteins. Neither the chemical identity of the radiolabelled species binding to tissue protein nor the incorporation of radiolabel into plasma protein was investigated. Although this mechanism cannot explain the covalent attachment of unmodified VPA to protein, the investigators noted that two inhibitors of glucuronidation decreased the initial rapid binding of radiolabel to hepatocytes, and suggested that acyl glucuronides of [^{14}C]VPA and [^{14}C]Δ^4-VPA could contribute to this phase.

Of 57 patient plasma samples tested, nine displayed a measurable antibody response to the covalent VPA–HSA adducts, though antibody titres in the ELISA were very low. There appeared to be no correlation between plasma concentration of VPA–protein adducts and the antibody titre. The low titres detected in our study suggest that VPA–protein adducts are only weakly immunogenic. VPA has achieved widespread use as a broad spectrum antiepileptic agent. Its most serious (though rare) side effect is severe, sometimes fatal, hepatotoxicity [44], but the evidence suggests drug/metabolite toxicity rather than drug hypersensitivity [44, 45]. Indeed, this agent seems not to have any significant history of hypersensitivity induction. It would be of considerable interest to determine whether carboxylic acid drugs known to induce hypersensitivity (e.g. some non-steroidal anti-inflammatory agents) induce higher titres of anti-adduct antibodies.

Note added in proof. As noted by one of the reviewers, it is possible that protein could be rendered immunogenic without being involved in adduct formation. Thus, strictly speaking, on the basis of the present data, it is appropriate to refer to "VPA-modified protein" and "anti-VPA-modified protein antibodies" in patients' plasma.

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