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Inhibition of the formation of 4-hydroxyandrostenedione glucuronide by valproate

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The steroid, 4-hydroxyandrostenedione (HAD) is a potent specific inhibitor of the estrogen synthetase enzyme complex, aromatase which is responsible for the conversion of androgens into estrogens [1, 2]. Clinical trials have shown HAD to be capable of suppressing plasma estradiol levels and producing tumour regression in postmenopausal patients with advanced breast cancer [3, 4]. Metabolism studies demonstrated that HAD is rapidly conjugated to the glucuronide and this is the principal metabolite in rats and humans [5, 6]. Extensive conjugation of HAD has been proposed as the reason for the inability to detect HAD in rat plasma using gas chromatography–mass spectrometry, despite a sensitivity down to 50 ng/ml [5]. The present investigation is an attempt to reduce the extent of this "adverse" metabolism. Taburet and Aymard [7] have shown that the drug, valproate (VPA), inhibits the glucuronide conjugation of parahydroxyphenobarbital by rat liver microsomes. Experiments are described below in which the effect of VPA on the conjugation of HAD is studied using rat hepatocytes. Plasma HAD levels and amounts of HAD glucuronide in bile were estimated in rats given HAD alone and HAD + VPA.

Materials and methods

Materials. [^{14}C]4-hydroxyandrostenedione was synthesized as described by Foster *et al.* [6]. Valproic acid and cyclohexane carboxylic acid were obtained from Aldrich Chemical Co Ltd (Gillingham, U.K.). For *in vitro* experiments valproic acid was neutralized with N NaOH to form sodium valproate whilst for the *in vivo* work sodium valproate was given in the form of a syrup, Epilim, containing 40 mg/ml. β-Glucuronidase, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim. Lipidex 5000™ and Sep-Pak G_{18} cartridges were obtained from Canberra Packard and Waters Assoc. (Milford, MA) respectively.

In vitro *studies.* Hepatocytes were isolated from the livers of phenobarbital treated male Wistar rats by a 2-step collagenase perfusion technique previously described [6]. Suspensions of hepatocytes (5×10^6 cells/ml) were incubated with 0.33 mM HAD labelled [6] with [^{14}C] (208 μCi/mmole) in the presence or absence of various concentrations of valproate (0.75 mM–3 mM). Samples of incubation mixture were extracted and processed as described [6] and finally the amounts of Phase I and Phase II metabolites determined after separation by thin layer chromatography and estimation of the relative proportion of radioactivity in each band using a Berthold TLC Linear Analyzer [6]. Identification of Phase I and Phase II metabolites of HAD has been described [6].

In vivo *studies.* Drugs were administered to the rats by gastric gavage, either 2.5 mg HAD in standard steroid vehicle or 2.5 mg HAD with varying amounts of valproate syrup containing 12.5–50 mg of sodium valproate. The rats were bled by cardiac puncture 1.5 hr later. For the 24 hr bile collection, the bile duct was cannulated under pentobarbitone anaesthesia immediately after HAD or HAD plus valproate were given orally.

Isolation of HAD from plasma. The plasmas (minimum vol of 2 ml) were passed through Sep-Pak cartridges and the dried eluates further purified by Lipidex column chromatography. Lipidex 5000 pre-equilibrated in 2,2,4-trimethylpentane : isopropanol (5:1) (TMP:IP) was packed into a glass column 420 mm × 4 mm. The elution profiles of androstenedione and HAD were pre-determined for each column using [^{3}H]androstenedione and [^{14}C]HAD. The dried eluate from the cartridges was dissolved in the solvent system and applied to the column and the appropriate fractions equivalent to HAD collected.

Isolation of HAD glucuronide from bile. Samples (24 hr) of bile were extracted with ethyl acetate at pH 1.5 to give a glucuronide fraction (mostly HAD glucuronide) which was hydrolysed with β-glucuronidase and the HAD so released estimated using the aromatase assay.

Estimation of HAD by aromatase assay. The pooled fractions containing HAD were transferred to assay tubes, taken to dryness and resuspended in 25 μl of ethanol. Activity of the aromatase enzyme from human placental microsomes was monitored by measuring the tritiated water formed from [^{3}H]androstenedione in the presence of a NADPH regenerating system consisting of NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase [8]. Each sample was assayed in duplicate over three time points. The results were plotted on a graph of product released against time of incubation. The resulting linear graph was utilised to determine the rate of enzymic reaction for each sample. The values were compared to control samples (normal plasma) and the per cent activity of control samples determined. A calibration curve (per cent activity of aromatase enzyme against concentration of HAD) was produced using normal plasma containing a known concentration of HAD and working up the samples as described. Quantification of HAD present in samples of unknown concentration was made by comparison of inhibitory activity with the calibration curve. The lower limit of sensitivity of this bioassay was 10 ng of HAD/ml.

Results and discussion

Addition of VPA to a suspension of rat hepatocytes metabolising [^{14}C]HAD caused a fall in the amount of radioactive label appearing in the conjugate-containing aqueous fraction whilst at the same time levels of [^{14}C] in the ethyl acetate fraction (un-metabolised HAD and phase I metabolites) were increased (Table 1). Further analysis of radiochromatograms (not shown) from this latter fraction revealed that although only small amounts of HAD

remained after a 15 min incubation period, these levels increased in a dose-related manner when increasing concentrations of VPA were added (Fig. 2). All the glucuronide peaks shown in Fig. 1d were decreased compared to the control (a) and lesser concentrations of VPA (b and c), the most marked changes being in the principal glucuronide, that of HAD itself.

Previous attempts to quantify HAD in rat plasma using a capillary gas chromatography–mass spectrometric assay had failed, even though a sensitivity of 50 ng/ml was achieved [5]. Therefore we used a bioassay (sensitivity 10 ng/ml) based on the inhibition of aromatase activity to investigate the possibility than an inhibition of HAD glucuronide formation *in vivo* would result in elevated plasma levels of HAD. Results in Table 2 suggest that this is indeed so. Because of the volume of blood required for each HAD determination (2 ml) serial blood sampling from the same animal was not feasible. Each plasma HAD value given in Table 2 is from a different rat. One and a half hours after the rats received HAD plus VPA levels of HAD measured in the plasma were more than twice those in rats given HAD alone.

These observations were further strengthened by the finding of reduced amounts of HAD glucuronide in bile from rats given HAD plus VPA (Table 3).

In conclusion, the rapid formation of HAD glucuronide by rat hepatocytes was inhibited in a dose dependent manner by sodium valproate. Increased HAD levels in plasma

Table 1. Effect of VPA on % total radioactivity in ethyl acetate (free steroids) and aqueous (conjugates) fractions after incubation of [^{14}C]HAD with rat hepatocytes

	% Radioactivity			
Conc of sodium valproate (mM)	15 min incubation		60 min incubation	
	Ethyl acetate	Aqueous fraction	Ethyl acetate	Aqueous fraction
0	51	30	10	75
0.75	61	21	19	67
1.5	71	17	25	59
3.0	78	15	33	47

The above results are representative of those obtained from experiments carried out in duplicate for each time period.

Table 2. Effect of VPA on HAD levels in plasma of rats 1.5 hr after oral administration of the drugs

Treatment	HAD ng/ml plasma-values for individual animals				$\bar{x}$	SEM
2.5 mg HAD	0	0	16	40	14	9.45
2.5 mg HAD + 12.5 mg VPA	0	16	24	40	20	8.33
2.5 mg HAD + 25 mg VPA	45	53	65		54	5.81*
2.5 mg HAD + 50 mg VPA	53	107	165		108	32.34*

* Values are significant according to the Mann–Whitney U-test. $P < 0.05$ compared to control.

Table 3. Effect of VPA on amounts of HAD (after hydrolysis of HAD glucuronide with β-glucuronidase) in 24 hr samples of rat bile

Treatment (drugs given orally)	HAD (μg) in 24 hr bile samples	% dose in 24 hr bile
2.5 mg HAD	760	30
2.5 mg HAD + 12.5 mg VPA	740	30
2.5 mg HAD + 25 mg VPA	675	27
2.5 mg HAD + 50 mg VPA	510	20

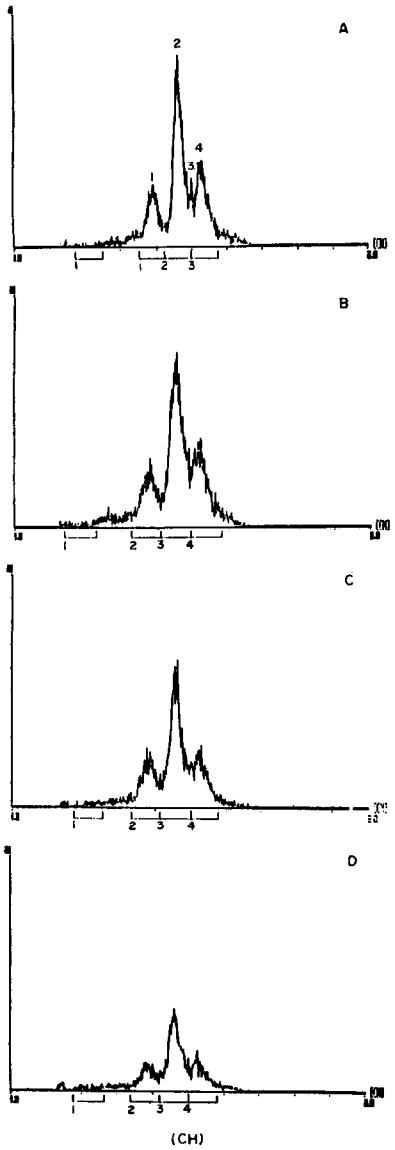

Fig. 1. Inhibition of glucuronide formation by VPA. Radiochromatograms of ethyl acetate fraction extracted at pH 1.5: (A) Controls; (B) 0.75 mM sodium valproate; (C) 1.5 mM sodium valproate; (D) 3 mM sodium valproate. Glucuronides (% present): (1) 3α-hydroxy-5β-androstan-4,17-dione (20%); (2) 4-hydroxyandrostene-3,17-dione (50%); (3) 2,4,17β-trihydroxyandrostan-3-one (6%); (4) 3β, 4β-dihydroxyandrostan-17-one (24%). Abscissa-distance travelled on TLC plate in cm. Ordinate—total counts picked up from track on TLC plate in 10 min.

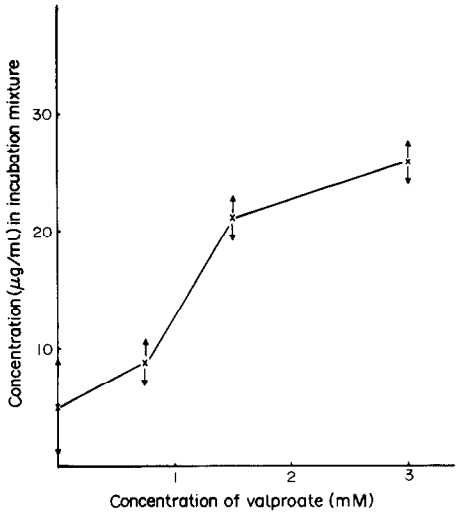

Fig. 2. Effect of VPA on amounts of substrate left after incubation of HAD with rat hepatocytes for 15 min.

together with decreased amounts of HAD glucuronide in the bile suggested that this inhibition of glucuronide formation may occur *in vivo* when valproate and HAD are co-administered.

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