Biochemical Pharmacology, Vol. 33, No. 10, pp. 1595–1600, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

AN INVESTIGATION OF THE EFFECTS OF PHENOBARBITONE ON THE PHARMACOKINETICS OF NORETHINDRONE IN THE RAT USING LIVER PERFUSION AND EVERTED GUT SACS

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(*Received* 28 *June* 1983; *accepted* 21 *November* 1983)

Abstract—Previous *in vivo* studies have suggested that phenobarbitone increases the first pass clearance of norethindrone in the rat by induction of enzymes both in the gut wall and liver. In the present study phenobarbitone caused an increase in both the production of highly polar ether-extractable metabolites and the conjugation of the steroid as it crossed the wall of the everted gut sac preparation. In addition, there was a marked increase in the uptake of norethindrone into the liver followed by increased phase I metabolism in the isolated perfused liver. As expected for a highly cleared drug, enzyme induction had no measurable effect on the terminal half-life of norethindrone in the perfused liver preparation.

Phenobarbitone interacts with oral contraceptive steroids in animals and humans [1, 2]. Studies in rats have shown that barbiturate causes changes in peak plasma concentration and the area under the curve (*AUC*) following oral administration and there was evidence of increased hepatic metabolism of norethindrone (Nor). It was suggested that phenobarbitone increased the gut wall metabolism of Nor and that this further contributed to the reduced peak height and *AUC*.

Using the isolated perfused rat liver, the extraction ratio and clearance of the drug can be determined by measuring the decrease in plasma concentration across the liver. The rat is an appropriate animal to study since it does not possess a specific sex steroid binding protein in plasma and has high hepatic levels of 4-hydrogenase, 3-hydroxysteroid dehydrogenase and hydroxylase enzymes which are known to be involved in the metabolism of steroids [3, 4]. This work, therefore, investigates the effect of phenobarbitone on the metabolism of Nor by the isolated perfused liver and everted sacs of ileum of the rat.

MATERIALS AND METHODS

Apparatus

The apparatus used for the perfusion of the isolated rat liver was similar to that of Miller *et al.* [5].

Perfusion media

Female, non-fasted Wistar rats weighing 300–400 g served as blood donors, the blood being obtained by cardiac puncture under ether anaesthesia into syringes containing approximately 250 units of heparin (Leo Laboratories, Middlesex, U.K.). Prior to use, the blood was filtered through a single layer of Kimwipes® (Kimberley-Clark, U.S.A.) and then blood (2 vols.) was diluted with Krebs–Henseleit buffer (1 vol.).

After dilution the haematocrit was reduced to approximately 25%. In all cases the haematocrit was maintained above 20%. The pH of the diluted blood was between 7.20 and 7.44.

All rats for use as blood donors were donated by ICI (Alderley Park, Cheshire, U.K.).

Experimental animals

One group (control) of female Wistar rats (200–300 g) received physiological saline while the other group was treated with phenobarbitone (40 mg/kg twice daily for 5 days, orally). Animals were operated upon on day 7.

Indices of induction

The microsomal protein and cytochrome P-450 contents of the livers from five control and five phenobarbitone-treated rats were measured by the methods of Lowry *et al.* [6] and Omura and Sato [7].

Operative technique

Rats were anaesthetized with ether and then pithed and artificially ventilated. The bile duct was cannulated with Portex tubing (PP 50). A glass cannula (o.d. 1.75 mm, i.d. 1.5 mm) was inserted into the portal vein and perfusion was started immediately this cannula was tied in place. The inferior vena cava was cannulated between the heart and liver with a glass cannula (o.d. 2.5 mm, i.d. 2.0 mm). The liver was then removed and placed in an organ chamber and allowed to equilibrate for approximately 30 min.

Prior to the start of the experiment, the flow rate was adjusted to 15 ml/min, the perfusion pressure being between 11 and 16 mmHg.

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Single pass perfusion

(i) *Experimental procedure.* Livers from control and phenobarbitone-treated rats were studied. The volume of perfusate introduced into the system was approximately 125 ml. Norethindrone [12.5 μg unlabelled steroid and 2 μCi [6,7-^{3}H]-(21.6 Ci/mmole; Radiochemical Centre, Amersham, Bucks., U.K.) in saline–ethanol, 6:1 v/v; 200 μl] was introduced into the portal vein as a bolus injection. The total perfusate leaving the liver via the hepatic vein was collected in 15 sec aliquots over a total time period of 5 min.

(ii) *Analysis of samples.* The blood samples were centrifuged and the plasma removed. Aliquots (50 μl) of each sample were removed and the total radioactivity was counted in micellar scintillant (NE260; Nuclear Enterprises, Scotland, U.K.; 4 ml). Free steroids were extracted from duplicate aliquots (200 μl) of alternate samples with diethyl ether (2 × 4 ml). The ether was evaporated and the tritium content of one duplicate counted directly. The other was analysed by thin-layer chromatography (TLC). Standard markers of Nor metabolites (5α-17α-ethinyloestrane-3β,17β-diol and 5β-17α-ethinyloestrane-3α,17β-diol; G. D. Searle & Co.) were also applied to the TLC plate. Thin-layer chromatograms (silica gel, Kodak Eastman, type 13181) were run in methylene chloride–diethyl ether (80:20, v/v). An unlabelled reference sample of Nor was added and visualized under UV light. Radioactivity on the TLC plates was detected by scraping horizontal bands (0.5 cm) into scintillation vials and counting. Authentic standard steroids were detected by spraying with an anisaldehyde/sulphuric acid reagent. The radioactivity remaining in plasma following the ether extraction was counted to determine the percentage of conjugates.

Recirculating procedure

(i) *Experimental procedure.* The total volume of perfusate introduced into the system was 125 ml in all cases. Norethindrone (12.5 μg) in saline–ethanol (6:1 v/v; 200 μl) was introduced into the reservoir. Samples (1 ml) were taken from the inflow and outflow cannulae at times between 0 and 90 min. At the end of the experiment, the liver was weighed, placed in ice-cold KCl (1.15%; pH 8.0) and homogenized. An aliquot (100 μl) was removed for estimation of Nor by radioimmunoassay [8].

(ii) *Calculation of pharmacokinetic parameters.* The perfusate-inflow concentrations were best resolved in all experiments into two exponential components. The half-life of the slow disposition phase ($t_{1/2\beta}$) was calculated by least-squares regression analysis and that of the fast disposition phase ($t_{1/2\alpha}$) by the method of residuals. The areas under the perfusate concentration–time curves for both inflow and outflow concentrations (AUC_{pre} and AUC_{post}) were calculated by the trapezoidal rule.

The mean extraction ratio (E_m) can be calculated from the equation:

$$E_m = \frac{(AUC_{pre} - AUC_{post})}{AUC_{pre}}$$

The hepatic clearance (Cl_H) is equal to the product of liver blood flow (Q) and the mean extraction ratio:

$$Cl_H = Q \times E_m$$

Intrinsic clearance (Cl_{int}) is calculated as:

$$Cl_{int} = \frac{Q \times E_m}{1 - E_m}$$

Distribution of norethindrone between rat erythrocytes and plasma

Duplicate blood samples (1 ml) were taken from a normal perfusion experiment at 10, 15, 20, 30 and 50 min and the plasma was separated from one duplicate by centrifugation. To the other was added an equal volume of sodium citrate (0.25% w/v) to cause lysis of the red blood cells. The Nor content of both samples was measured by radioimmunoassay.

Metabolism of norethindrone by everted gut sacs of rat ileum

The rats (control and phenobarbitone-treated) were killed by a blow on the neck and the ileum was removed, everted and made into three sacs of equal length [9]. The sacs were filled with human male plasma (2 ml) and then incubated with Krebs–Henseleit buffer (10 ml) containing 0.1 μCi tritiated Nor under an atmosphere of O_2–CO_2 (95%:5%) for 1 hr at 37°. One sac from each rat was incubated in the presence of the metabolic inhibitor thiomersal (0.5% w/v). At the end of the incubation period duplicate aliquots (200 μl) of the sac contents were extracted twice with diethyl ether (2 ml) and the radioactive content of one of each of the duplicate aqueous and organic phases was measured. The other extract was evaporated and the metabolites were identified by TLC as described previously.

RESULTS

Assessment of condition and performance of the isolated perfused rat liver

Flow rate remained constant during each study and portal pressure was stable. There was no significant difference between the mean flow rate for phenobarbitone-treated and control livers (phenobarbitone-treated 1.36 ± 0.29; control 1.37 ± 0.19 ml/min per g liver; ±S.D.). All livers were uniformly red in colour and there was no oedema. An irregular surface of the liver indicated air bubbles had entered the system and such livers were discarded. The rate of bile production was approximately 0.4 ml/hr, although in some experiments there was a decrease after 90 min of perfusion.

Indices of enzyme induction

In the livers of rats administered phenobarbitone, there was a significant increase in microsomal protein (control 80.8 ± 8.56, treated 121.4 ± 3.27 mg/100 g body wt; $P < 0.05$), and in the cytochrome P-450 content (control 0.67 ± 0.04, treated 0.94 ± 0.07 nmole/mg protein, $P < 0.05$).

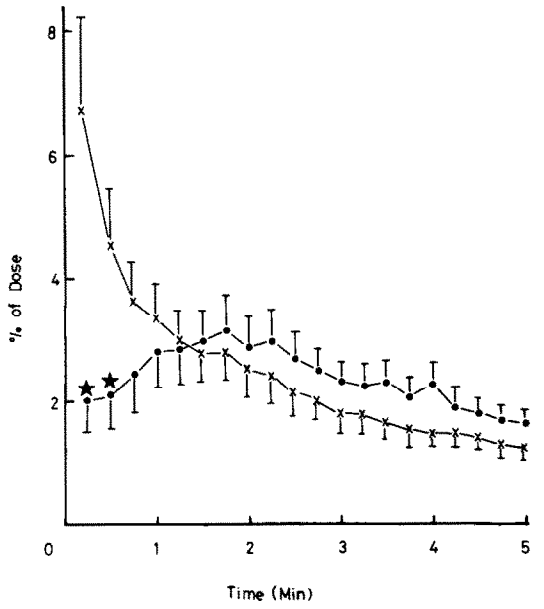

Fig. 1. Total radioactivity leaving an isolated perfused liver from control and phenobarbitone-treated rats after administration of norethindrone into the hepatic portal vein. Each point is the mean of five experiments; vertical lines represent ± S.E.M. (control ×——×; phenobarbitone ●——●). *$P < 0.05$.

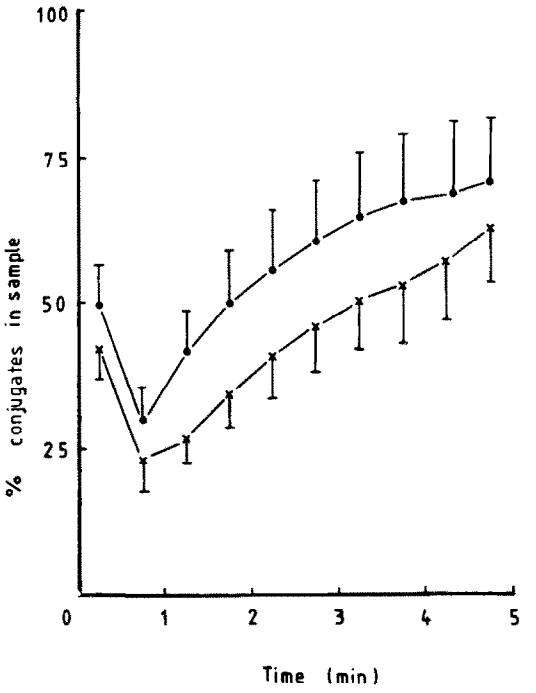

Fig. 2. The effect of phenobarbitone treatment on the appearance of non-ether-extractable metabolites of norethindrone in the perfusate of the isolated rat liver after administration of norethindrone into the hepatic portal vein (× control; ● phenobarbitone).

Single-pass perfusion experiments

The total radioactivity leaving the liver was significantly lower during the first 30 sec of perfusion of livers from phenobarbitone-treated rats as compared to the control group ($P < 0.05$). At the time points after this, there was no significant difference in the total radioactivity or the percentage of conjugates in the two groups (Figs. 1 and 2).

TLC of the ether-extractable metabolites yielded three distinct areas of radioactivity: I (polar metabolites, R_f 0.09); II (ring A reduced metabolites, R_f 0.38–0.46) and III (norethindrone, R_f 0.53). Ring A reduced metabolites could not be adequately separated to provide identification of the individual epimers. After 15 sec of perfusion, the percentage of unchanged Nor was significantly lower ($P < 0.05$) and the ring A reduced metabolites significantly higher ($P < 0.01$) in the phenobarbitone-treated group (Fig. 3). At all time intervals after this, there was a significant increase in the percentage of polar metabolites and a significant decrease in the unchanged Nor and ring A reduced metabolites in the phenobarbitone-treated group.

At the end of the 5 min perfusion period, the percentage of administered radioactivity remaining in the liver was 49.4 ± 8.6% (mean ± S.E.M.) in the control group and this was not significantly different from that of 51.4 ± 7.0% in the phenobarbitone-treated group.

Recirculating perfusion

Inflow concentration–time curves for Nor, gave biexponential curves in all cases (Fig. 4). The mean perfusate concentration 2 min after administration of Nor was 88.6 ± 4.2 ng/ml (mean ± S.E.M.) in the control group and was not significantly different from that obtained in the treated group (108 ± 12.4 ng/ml). All pharmacokinetic parameters are summarized in Table 1 and phenobarbitone had no significant effect on any of these parameters. Nor remaining in the liver at the end of the perfusion was less than 0.25% of the administered dose in all cases.

Distribution of norethisterone between rat erythrocytes and plasma

Radioimmunoassay of plasma and haemolysed whole blood indicated that the Nor concentrations of both were very similar (Table 2).

Metabolism of norethindrone by everted sacs of rat ileum

The radioactivity in the contents of sacs from control and phenobarbitone-treated rats incubated with [^{3}H]Nor are shown in Table 3. There was no significant difference in metabolism between the three portions of the ileum, therefore, a mean value for the whole ileum was taken for each animal. When thiomersal was present, metabolism was completely inhibited and all the radioactivity was present as parent steroid. Phenobarbitone treatment significantly increased the non-ether-extractable radioactivity (conjugates) present in the gut sac contents. Phenobarbitone treatment decreased the percentage of ring A reduced metabolites and Nor, and increased the percentage of polar metabolites (Table 3).

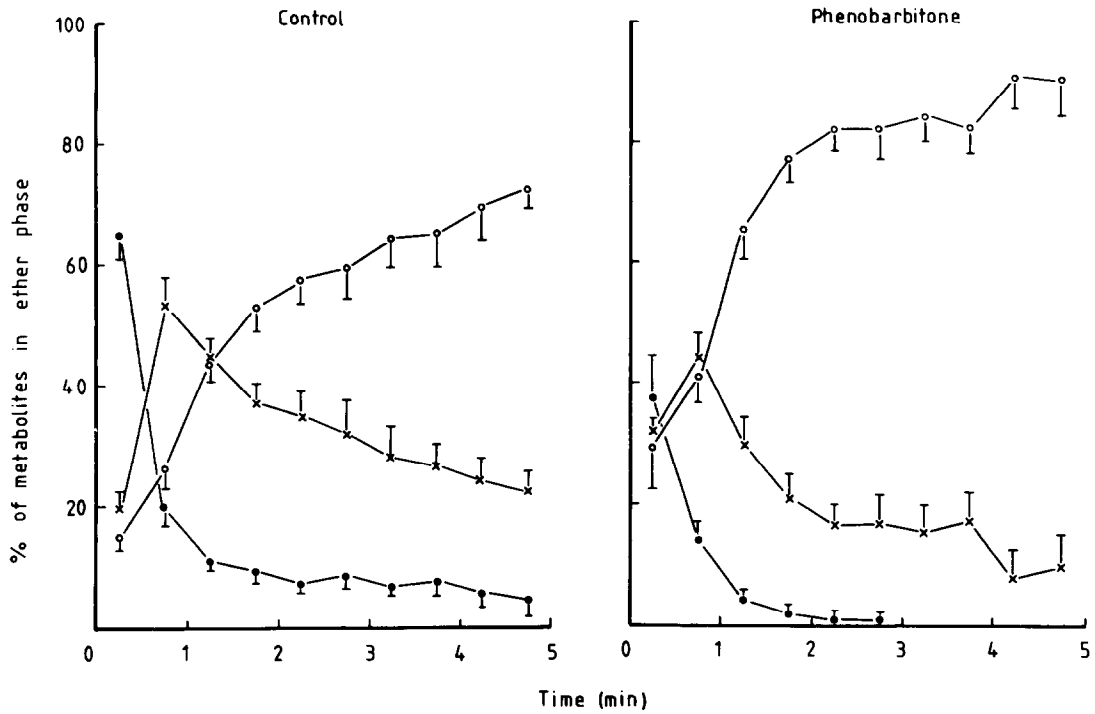

Fig. 3. The effect of phenobarbitone treatment on the relative concentrations of norethindrone and its ether-extractable metabolites in the perfusate of the isolated rat liver (● norethindrone; × tetrahydro-reduced metabolites; ○ polar metabolites).

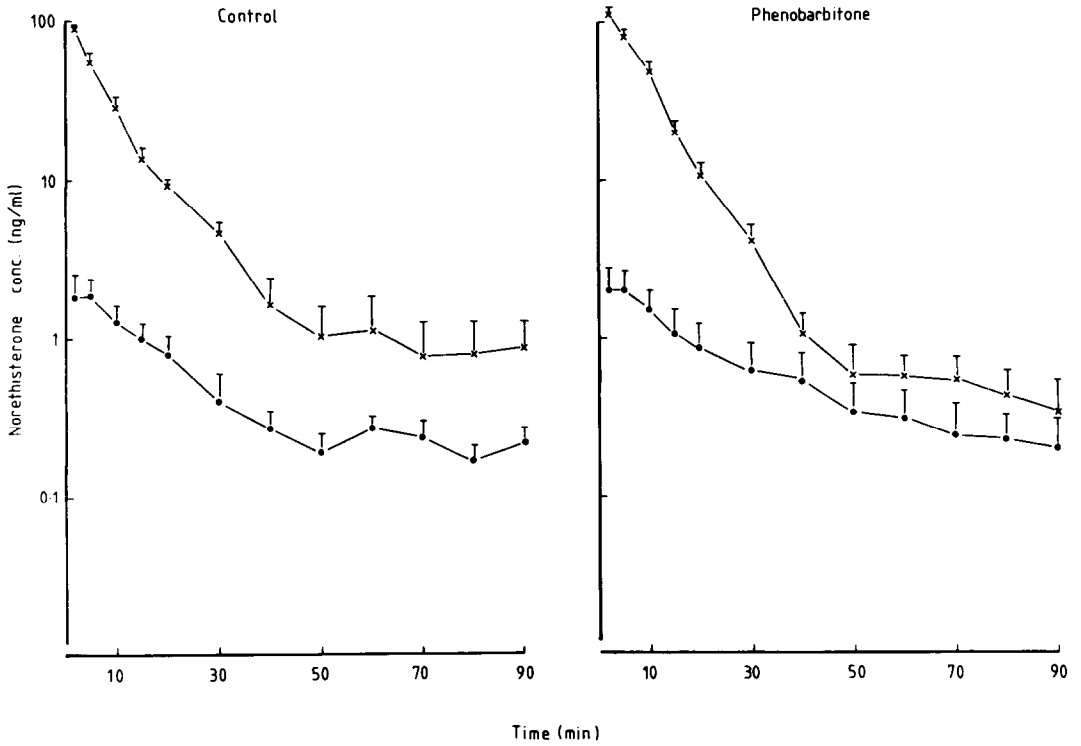

Fig. 4. Norethindrone concentration in samples of perfusate taken from the hepatic portal vein (×——×) and the hepatic vein (●——●) after introduction of 12.5 μg norethindrone into the reservoir of an isolated perfused liver from control and phenobarbitone-treated rats.

Table 1. Effect of phenobarbitone on pharmacokinetic parameters of norethindrone administered into the reservoir of isolated perfused rat livers in a recirculating system

	Control (n = 5)	Phenobarbitone (n = 4)
$t_{1/2\alpha}$ (min)	4.44 ± 0.54	4.90 ± 0.3
$t_{1/2\beta}$ (min)	43.9 ± 5.5	44.3 ± 10.7
AUC (ng/ml·min) pre liver	917 ± 49	1148 ± 95
AUC (ng/ml·min) post liver	46.1 ± 10.6	54.3 ± 22.8
Mean extraction ratio	0.95 ± 0.01	0.96 ± 0.02
Hepatic clearance (ml/min)	14.2 ± 0.2	14.4 ± 0.3
Intrinsic clearance (ml/min)	360 ± 103	664 ± 301

Means ± S.E.M.

Table 2. Norethindrone concentrations in rat whole blood and plasma

Time of sample (min)	Norethindrone concentration (ng/ml) Rat whole blood	Rat plasma
10	45.4	41.8
15	14.2	15.1
20	12.9	10.3
30	8.7	8.9
50	2.1	2.1

DISCUSSION

The bile flow was within the limits of 300–600 μl/hr described by other workers in the isolated perfused rat liver [10–12].

In common with many other lipophilic drugs, Nor was found to be able to pass freely into rat erythrocytes and, therefore, Nor was assumed to distribute rapidly throughout the blood.

The metabolism of Nor by the rat liver appears to be very rapid, with metabolites being released from the liver during the first 15 sec of perfusion. Freudenthal *et al.* [13] studied the metabolism of norethynodrel and they showed a similar pattern of metabolite formation to that in the present study. They suggested that the polar metabolites present in large quantities are polyhydroxylated metabolites. The present study showed an increase in the rate of formation of polar metabolites after treatment with phenobarbitone, which is known to increase hydroxylation of drugs including steroids by induction of the microsomal enzymes [14]. The reduced output of ring A reduced metabolites is probably due to such steroids being further metabolized to polyhydroxylated products. No increase in conjugate formation was detected after phenobarbitone, but as conjugates of Nor are rapidly excreted in the bile [15] there may have been increased elimination of conjugates by this route.

"X" and "Y" proteins are molecules which are thought to be associated with the transfer of drugs from plasma into the liver cell and which are known to bind hormones [16]. The concentration of "Y" is increased after phenobarbitone administration and in the present study we have shown that the initial uptake of Nor after a bolus portal injection is increased by phenobarbitone. This markedly increased initial uptake may, therefore, be due to increased binding proteins in the liver.

Recirculating perfusion

The initial decline in concentrations of Nor in the perfusate is very rapid due to continued dilution within the apparatus and uptake by the liver. The initial extraction ratio is very high and clearance of the steroid approaches hepatic blood flow. This rapid disappearance could be due to an efficient uptake mechanism by the liver and/or metabolism. Back *et al.* [17] have suggested there is an uptake process for Nor in the rat similar to that suggested for propranolol by Shand and Ragno [18].

Table 3. Norethindrone metabolites present in ether extracts of the contents of everted rat ileum

	Control (%)	Phenobarbitone (%)
Total ether-extractable radioactivity	93.6 ± 0.63	80.1 ± 3.76**
Norethindrone	15.4 ± 2.73	7.98 ± 1.81*
Ring A reduced metabolites	72.8 ± 1.62	49.8 ± 3.42**
Polar metabolites	5.4 ± 2.29	22.4 ± 3.73**

n = 5; means ± S.E.M.; *$P < 0.05$; **$P < 0.05$.

Phenobarbitone administration had no significant effect on any pharmacokinetic parameter. These observations agree with the Wilkinson and Shand model [19] of enzyme induction which suggests that induction will have relatively little effect on the intravenous profile of highly cleared drugs such as Nor. However, this model would predict changes in extraction ratio and intrinsic clearance. The study demonstrates the extreme difficulty in ever providing a practical demonstration of a change in Cl_{int} for a highly extracted drug. The problem arises because the calculation of Cl_{int} depends critically on the denominator $1-E_m$ where E_m is close to unity. The result of subtraction between two similar figures yields an excessively variable result when one of the values is derived from experimental data. The problem is clearly seen in Table 2 where the measured Cl_{int} is almost doubled in the phenobarbitone-treated animals and yet the result is not statistically significant because of the very high values for S.E.M.

Metabolism of norethindrone by everted gut sacs of rat ileum

The metabolism of Nor was not an artefact of the system as thiomersal, a metabolic inhibitor, completely abolished the formation of all metabolites. Conjugation and the formation of polar hydroxylated metabolites were both increased by phenobarbitone administration. Increased conjugation by the gastrointestinal tract after phenobarbitone treatment has been previously reported *in vivo* by Back *et al.* [2].

Overall conclusions

Norethindrone has a large first pass effect *in vivo* in the rat [17] after oral administration. After phenobarbitone administration there is a decrease in the area under the curve and peak height with no change in the half-life of the steroid [2]. The present work indicates that this decrease in the plasma concentration of Nor has two components, increased conjugation and production of highly polar metabolites (probably hydroxylation) by the gut, and increased uptake and metabolism by the liver.

Acknowledgements—We are grateful for financial assistance from the Mersey Regional Health Authority, The Wellcome Trust and the World Health Organisation.

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