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THE EFFECT OF ANTIBIOTICS ON THE ENTEROHEPATIC CIRCULATION OF ETHINYLESTRADIOL AND NORETHISTERONE IN THE RAT

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SUMMARY

Seventy-one percent of a given dose of [^{3}H]-ethinylestradiol ([^{3}H]-EE_2) and 76.0% of a given dose of [^{3}H]-norethisterone ([^{3}H]-N) were excreted in the bile of female rats in 4 h. The majority of radioactivity appeared in the glucuronide fraction. Characterization of the hydrolysed glucuronide fraction obtained after administration of [^{3}H]-EE_2 gave evidence that approximately 10.0% of the administered dose may be directly conjugated to form ethinylestradiol glucuronide. In contrast, there was no evidence of direct conjugation of norethisterone.

In a linked rat preparation 15.4% of the given dose of [^{3}H]-EE_2 was excreted in the bile of control recipient rats in 7 h; this was reduced to 5.2% in neomycin pretreated and 6.0% in ampicillin pretreated animals. With [^{3}H]-N, 13.2% of the dose was excreted in the bile of control recipient rats in 7 hours and this was reduced to 3.6% in neomycin pretreated and 3.9% in ampicillin pretreated animals.

These results show that antibiotic treatment reduces the enterohepatic circulation of synthetic steroids in the rat.

INTRODUCTION

There is increasing evidence that certain commonly prescribed drugs may interact with steroid contraceptives thereby reducing contraceptive efficacy and it has been suggested that such an interaction may occur with antibiotics. In 1973 Hempel, Bohm, Carol and Klinger[1] reported pregnancies in 2 patients taking contraceptive steroids who had also been receiving chloramphenicol and sulphamethoxypyradizine and Dossetor[2] reported pregnancies in 3 patients who were also taking ampicillin.

Synthetic oestrogens and progestogens undergo extensive biliary excretion both in man and laboratory species [3, 4], principally as glucuronide conjugates. Animal studies have established that the elimination of synthetic sex hormones may be a relatively slow process and this is largely attributable to enterohepatic circulation (EHC)—[4–7]. Since the gut microflora are a major source of hydrolytic enzymes, notably β-glucuronidase [8, 9] treatment with antibiotics may be expected to interfere with the hydrolysis of steroid conjugates and thereby increase the rate of steroid elimination from plasma. Brewster, Jones and Symons[10] demonstrated that neomycin reduced the biliary excretion of radioactivity after intraduodenal administration of labelled conjugates of mestranol. The present work was designed to study the effect of ampicillin and neomycin on the EHC of ethinylestradiol (EE_2) and norethisterone (N) in the rat.

MATERIALS AND METHODS

Radioactive materials

[6,7(*n*)-^{3}H]-Ethinylestradiol (45 Ci/mmol) was obtained from the New England Nuclear Corporation, Germany. [6,7(*n*)-^{3}H]-Norethisterone (21.6 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England. After evaporation of the organic vehicle under nitrogen the steroids were redissolved in a mixture of 0.9% saline–ethanol (9:1, v/v).

Animals

Female rats of the Wistar strain (160–300 g) were housed in groups in cages in well ventilated rooms at a temperature of approximately 24°C. The animals were allowed unrestricted access to food and water.

Thin layer chromatography

Thin layer chromatograms (t.l.c. aluminium sheets, Silica gel 60/Kieselguhr F_{254}; 20 × 20 cm; Merck; Darmstadt) were activated at 100°C for 60 min. The following solvent systems were used: A. methylene chloride diethyl ether (80:20, v/v); B. chloroform–methanol (97:3, v/v); C. methylene chloride–diethyl ether (80:20, v/v) followed by methylene chloride–ethyl acetate (95:5, v/v).

Detection of radioactive metabolites

Radioactivity on the t.l.c. plates was detected by scraping horizontal bands (0.5 cm width) individually into scintillation vials, adding scintillation fluid and

counting [11]. Authentic standards were run in each solvent system and were detected by spraying with an anisaldehyde–sulphuric acid reagent.

Biliary excretion of steroids

Rats were anaesthetized with urethane (14% w/v in 0.9% saline; 10.0 ml/kg, i.p.). Polyethylene catheters were inserted into a femoral vein, and the common bile duct. [^{3}H]-EE_2 (10 μCi/kg; 10 μg/kg) or [^{3}H]-N (10 μCi/kg; 125 μg/kg) was injected i.v. and bile samples collected in preweighed glass vials at 30 min intervals.

The radioactive content of bile (50 μl) was determined by liquid scintillation spectrometry [11]. From some aliquots of bile "free" steroids were extracted by shaking with ether (5 × volume) for 30 min. The resulting extract was evaporated to dryness, and after redissolving in methanol (20 μl) the radioactive content was determined. Further aliquots of bile were adjusted to pH 5.0 (0.1 M HCl) and added to β-glucuronidase (Ketodase, Warner Chilcott; 500 Fishman units per 10 μl bile). After incubation at 37°C for 2 h (previously found to be the optimum conditions) 'free' steroid was extracted and the radioactivity determined. Control specimens were prepared by adding β-glucuronidase to acidified bile containing glucaro (1 → 4) lactone (5 mM; Calbiochem).

Identification of parent steroids

(a) *Ethinylestradiol.* Bile was collected over a period of 4 h from 3 anaesthetized rats following i.v. administration of [^{3}H]-EE_2. After pooling the samples "free" steroids were extracted and the bile treated with β-glucuronidase as previously described. Ether extracts of the hydrolysate were evaporated to dryness, redissolved in methanol and subjected to t.l.c. in system B; four radioactive peaks were located. Peak I (at origin), Peak II (R_f = 0.10) and peak IV (R_f = 0.62) were not examined further. Peak III (R_f = 0.47) had the same mobility as ethinylestradiol. This peak was eluted and divided into three parts. One part was rechromatographed in system A and a single peak with the same mobility as EE_2 was seen. A second part was acetylated by adding 0.15 ml of dry pyridine–acetic anhydride solution (2:1, v/v) and leaving at 60°C for 2 h. On rechromatographing in solvent system B two peaks were seen corresponding to the authentic mono- and di- acetates of EE_2. The third part was crystallized to constant specific activity (Table 2).

(b) *Norethisterone.* Bile samples were collected from 3 rats over a period of 4 h following the i.v. administration of [^{3}H]-N. 'Free' steroids were extracted and the glucuronide fraction hydrolysed. Ether extracts of the hydrolysate were evaporated to dryness, redissolved in methanol and subjected to t.l.c. in systems A, B and C. The aim of the experiment was to ascertain the presence or absence of norethisterone in the hydrolysate and not to make a definitive characterization of other metabolites. The best separation of standard norethisterone from other standards was found in system A (Table 3). Radioactivity was found in 3 peaks in system A. Peak I (R_f = 0.08) and peak II (R_f = 0.29) were not examined further. Peak III had the same mobility as 19-nor-17α-pregn-4-en-20-yne-3β, 17-diol (4-en,3β-diol, R_f = 0.43) and 19-nor-5α, 17α-pregn-20-yne-3α, 17-diol (5α,3α-diol, R_f = 0.46). This peak was eluted, acetylated and rechromatographed in system A. Acetylation did not effect separation of 4-ene-3β-diol and 5α, 3α-diol. There was no radioactive peak approximating to authentic norethisterone acetate.

Effect of antibiotics on the EHC and EE_2 and N

The method chosen to study the EHC was to use the "linked animal" preparation previously described by Ladomery, Ryan and Wright[12]. In this preparation the bile duct cannula from a "donor" rat is inserted into the duodenum of a "recipient" rat. For experiments involving antibiotics, recipient rats were pretreated with ampicillin or neomycin sulphate (100 mg/body weight/day for 5 days; orally).

Rats were anaesthetized as previously described and polyethylene catheters inserted into the femoral vein and common bile duct of the donor rat and the common bile duct of the recipient rat. The bile duct cannula from the donor rat was tipped with a hypodermic needle to facilitate entrance into the duodenum of the recipient rat. After injection (i.v.) of the labelled steroid to the donor rat, bile was collected from the recipient rat in preweighed glass vials over a period of 7 h. Total radioactivity was determined.

RESULTS

Biliary excretion

The biliary excretion of [^{3}H]-EE_2 and [^{3}H]-N is shown in Figs. 1 and 2. Seventy-one percent of the

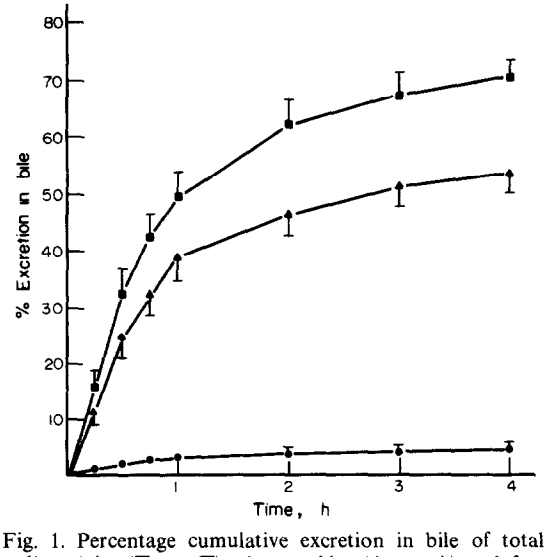

Fig. 1. Percentage cumulative excretion in bile of total radioactivity (■——■), glucuronides (▲——▲) and free steroids (●——●) following i.v. administration of [^{3}H]-ethinylestradiol (10 μCi/kg; 10 μg/kg). Each point is mean ± S.E.M. of 4 experiments.

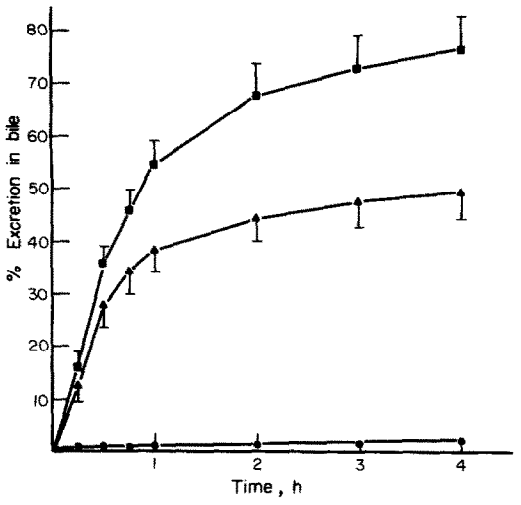

Fig. 2. Percentage cumulative excretion in bile of total radioactivity (■——■), glucuronides (▲——▲), and free steroids (●——●) following i.v. administration of [^{3}H]-norethisterone (10 μCi/kg; 125 μg/kg). Each point is mean ± S.E.M. of 4 experiments.

Table 1. Resolution of the hydrolysed glucuronide fraction from bile collected over a period of 4 h following i.v. administration of [^{3}H]-EE_2

Metabolite	% Of ether extractable radioactivity after hydrolysis
M1 (origin)	5.0
M2 (polar metabolites)	46.2
*M3 (EE_2)	19.9
M4 (unknown)	28.9

* Identified in several chromatographic systems, by chemical transformation and by recrystallization.

Table 2. Determination by crystallization of the identity of [^{3}H]-EE_2 isolated by t.l.c. from extracts of glucuronidase treated bile

Crystallization	Specific activity (d.p.m./mg)		
Methanol	1614	1686	1569
Cyclohexane–ethyl acetate	1514	1474	1477

given dose of EE_2 was excreted in bile in 4 h; the majority (53.9% of dose) was glucuronide conjugates. Only 4.6% was readily extracted as "free" steroids. The glucuronide fraction of bile samples collected for 4 h was examined to see whether or not EE_2-glucuronide was present. Although the majority of radioactivity was not characterized (Table 1) there was evidence of EE_2 in the hydrolysed fraction. This was further confirmed by recrystallization to constant specific activity.

Seventy-six percent of the given dose of N was excreted in bile in 4 h: the majority (49.2% of the dose) as glucuronide conjugates. Only 1.8% was readily extracted as free steroid. There was no evidence under the experimental conditions described of norethisterone being present in the glucuronidase-treated fraction of bile (Table 3). The majority of radioactivity (61.2%) in this fraction appeared as polar metabolites.

Antibiotic treatment

The effect of antibiotic treatment on the biliary excretion of radioactivity in recipient rats after i.v. administration of [^{3}H]-EE_2 or [^{3}H]-N to donor rats is shown in Figs 3 and 4. Ampicillin and neomycin pretreatment markedly reduced the amount of steroid appearing in the bile of recipient rats. With EE_2, 15.4% of the dose was excreted in the bile of control recipient rats in 7 h; this was reduced to 5.2% in neomycin pretreated and 6.0% in ampicillin pretreated animals. With N, 13.2% of the dose was excreted in bile of control recipient rats in 7 h and this was reduced to 3.6% in neomycin pretreated and 3.9% in ampicillin pretreated animals.

DISCUSSION

It has previously been shown that both EE_2 [6] and N [7] are extensively excreted in the bile of rats,

Table 3. Resolution of the hydrolysed glucuronide fraction from bile collected over a period of 4 h following i.v. administration of [^{3}H]-N

Metabolite	R_f in solvent system A	% Of ether extractable radioactivity after hydrolysis
*Polar metabolites	0.08	61.2
*Other	0.29	1.1
5β,3α-diol	0.37	—
5α,3β-diol	0.39	—
4-ene,3β-diol	0.43	37.7
**5α,3α-diol	0.46	
† Norethisterone	0.53	—

* Identified by chromatography in systems A, B and C. ** Identified by chromatography in systems A, B and C and by chemical transformation (acetylation). † No evidence of norethisterone.

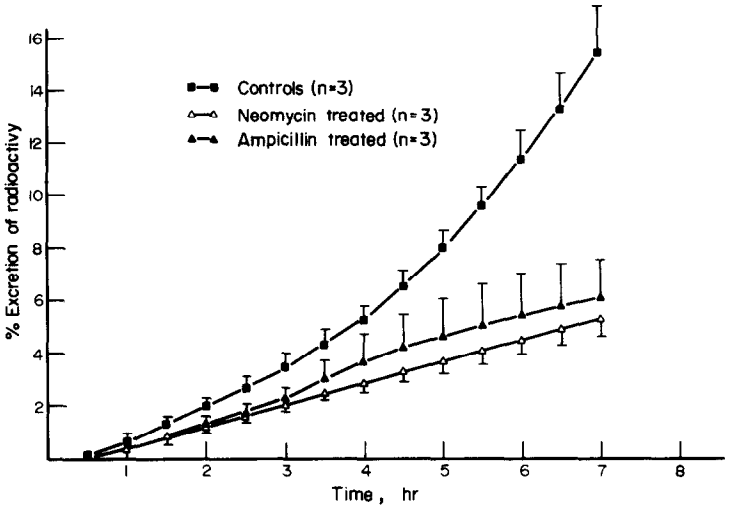

Fig. 3. Excretion of radioactivity in the bile of control (■——■), neomycin treated (△——△) and ampicillin treated (▲——▲) recipient rats. [^{3}H]-ethinylestradiol (10 μCi/kg; 10 μg/kg) administered i.v. to donor rats. Each point is mean ± S.E.M. of 3 experiments.

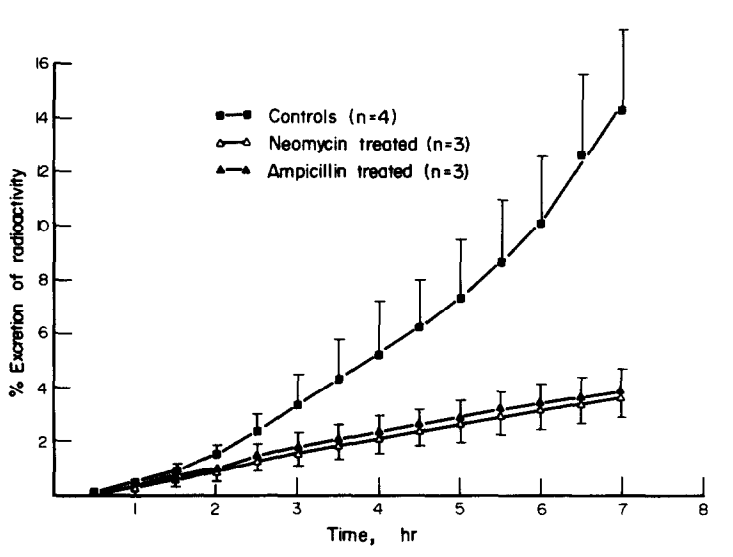

Fig. 4. Excretion of radioactivity in the bile of control (■——■), neomycin treated (△——△) and ampicillin treated (▲——▲) recipient rats. [^{3}H]-norethisterone (10 μCi/kg; 10 μg/kg) administered i.v. to donor rats. Each point is mean ± S.E.M. of at least 3 experiments.

predominantly as glucuronides. The present experiments confirm this and demonstrate that for both steroids biliary excretion is quantitaively of greatest significance during the first hour after drug administration.

Since only a small percentage of biliary radioactivity was freely extractable, and polar conjugates are not well absorbed from the intestine, it is evident that the radioactivity appearing in the bile of recipient rats in the linked preparation is virtually all derived from hydrolysis of conjugates in the intestine.

Although glucuronidase is present in mammalian gastrointestinal tissue at all levels [13] enzyme of bacterial origin is considered of greater importance in the EHC of both natural and foreign compounds [9]. In the present experiments antibiotic pretreatment of recipient rats considerably reduced the biliary elimination of the steroids and strongly suggests that under such conditions there is marked disruption of the EHC. Complete inhibition of recirculation was however not attained. The biliary excretion of radioactivity in antibiotic treated recipient rats may indicate either incomplete elimination of intestinal microorganisms or hydrolysis of conjugates by intestinal tissue enzyme.

In the rat there is an important difference between EE_2 and N in the qualitative pattern of biliary metabolites. We have found no evidence that norethisterone itself is conjugated; in contrast, EE_2-glucuronide appears in bile and accounts for approximately 10% of the injected dose.

It has previously been shown that there is a reduction in both urinary and plasma estriol levels in pregnant women during ampicillin or phenoxymethylpeni-

cillin treatment [14,15], and of urinary estriol levels during neomycin treatment [16]. It was suggested [16] that antibiotics can interfere with the normal enterohepatic circulation and urinary excretion of estrogens during pregnancy.

The present work has demonstrated that antibiotic treatment will reduce the EHC of synthetic steroids in the rat. In man, both N [3, 17] and EE_2 [3, 18] are extensively metabolized and excreted as glucuronides with evidence of both N-glucuronide and EE_2-glucuronide being formed. It is therefore reasonable to assume that antibiotic treatment will interfere with enterohepatic circulation. In the clinical context, the important issue is whether or not EHC of either one or both of the components of a combined oral contraceptive steroid preparation contributes to the therapeutic blood level and contraceptive efficacy. Contraceptive failure is known to occur with concurrent antibiotic administration [1, 2] and this suggests that EHC is not only of pharmacological interest but is of clinical importance.

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