Journal of Chromatography, 423 (1987) 63–73
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3904

MEDROXYPROGESTERONE ACETATE- AND ETHINYLESTRADIOL-INDUCED CHANGES IN BILIARY BILE ACIDS OF THE RAT STUDIED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received March 12th, 1987; revised manuscript received July 6th, 1987)

SUMMARY

The effects of subcutaneous administration (5 mg/kg per day) for seven days of medroxyprogesterone acetate (MPA) or 17α-ethinylestradiol (EE) on bile flow, total bile acid output and individual biliary acids have been studied in adult male Wistar rats. Biliary bile acid composition was quantitated by a simple isocratic high-performance liquid chromatographic technique using a C_{18} reversed-phase radial compression column and refractive index detection. This method revealed that muricholic acids, analysed as taurine and glycine conjugates, constituted a higher proportion of biliary bile acids in the rat than previously observed with gas chromatographic techniques. Marked cholestasis was produced by EE while MPA had little effect on bile flow or total bile acid output. Despite this, both steroids significantly increased the proportion of taurine-conjugated muricholic acids relative to taurocholic acid, although the estrogen had the more pronounced effect. Further study of the hepatobiliary consequences of high doses of MPA would seem warranted in view of the important use of this progestogen for cancer therapy.

INTRODUCTION

The contraceptive estrogen 17α-ethinylestradiol (EE; 17α-ethinyl-1,3,5(10)-estratriene-3,17β-diol) is a well established cholestatic agent for experimental studies [1]. Cholestasis is accompanied by impaired biliary bile acid secretion in the intact rat [2], and isolated hepatocytes from rats with EE-induced cholestasis exhibit a selective reduction in the efflux of conjugated bile acids [3]. EE has been shown to impair the conjugation of exogenous cholic acid and to increase the percentage of 6β-hydroxylated bile acids in the bile of rats at the expense of cholic acid [4]. An increased proportion of cholic acid and decreased proportion

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0378-4347/87/$03.50

of chenodeoxycholic acid have been found in gallbladder bile of women taking oral contraceptives, although the type and duration were not specified [5]. In a later study women taking an oral contraceptive containing ethinylestradiol-3-methyl ether (Mestranol) were found to have increased biliary cholesterol saturation and lithogenic index which was associated with lower rates of biliary secretion and enterohepatic circulation of bile acids [6].

While EE, or other synthetic estrogens, are only administered in small doses, mostly for contraceptive purposes, medroxyprogesterone acetate (MPA: 17α-hydroxy-6α-methyl-4-pregnene-3,20-dione 17α-acetate) and related progestogens are extensively used in high doses as cancer chemotherapeutic agents. Contraceptive doses of MPA have been reported to impair hepatic function in women [7]. Recently MPA was found to be a potent effector of 3α- and 3β-hydroxysteroid dehydrogenases and a repressor of 5α-reductase in vitro, while these effects were abolished by simultaneous administration of estradiol [8]. However, the effects of MPA on bile flow and biliary bile acids have not been studied. In this report these effects are compared with those observed with EE. The application of a simple high-performance liquid chromatographic (HPLC) procedure for the analysis of the major bile acids is presented.

EXPERIMENTAL

Reagents and materials

EE, MPA and propylene glycol (PG; 1,2-propanediol) were obtained from Sigma (Poole, U.K.). [1-^{14}]glycocholic acid (51 Ci/mol) was from Amersham International (Amersham, U.K.). The following taurine (T)- and glycine (G)-conjugated and unconjugated bile acids (5β-cholan-24-oic acids) were used (hydroxyl substitution indicated): β-murichollic (β-MC; 3α,6β,7β); hyocholic (HC; 3α,6α,7α); cholic (C; 3α,7α,12α); ursodeoxycholic (UDC; 3α,7β); hyodeoxycholic (HDC; 3α,6α); chenodeoxycholic (CDC; 3α,7α); deoxycholic (DC; 3α,12α); lithocholic (LiC; 3α). Keto bile acids with the following substitutions were also used: 3α-ol-7-one; 3,7-dione; 3,12-dione; 3,7,12-trione (dehydrocholic acid). Conjugates of β-MC and HC and the keto bile acids were prepared as reported by Lack et al. [9]. All bile acid standards were obtained from Steraloids (Croyden, U.K.), Calbiochem (Bishops Stortford, U.K.) or Sigma. Chromatographically purified 3α-hydroxysteroid dehydrogenase (3α-HSD; EC 1.1.1.50), partially purified 7α-hydroxysteroid dehydrogenase (7α-HSD; EC 1.1.1.51) and *Clostridium perfringens* acetone powder (type IV strain ATCC 13124) were from Sigma; β-nicotinamide-adenine dinucleotide from Boehringer (Lewes, U.K.); HPLC-grade methanol from Rathburn Chemicals (Walkerburn, U.K.); and all other chemicals, including Scintran Cocktail T scintillation fluid and solvents for thin-layer chromatography (TLC) from BDH (Poole, U.K.). Precoated TLC silica gel plates (Sil G25, Silgur and nano-grade) were obtained from Camlab (Cambridge, U.K.). Aqueous solutions of the mobile phase for HPLC were prepared as previously reported [10]. Purity of bile acid standards was evaluated by HPLC and the 3α-HSD assay (see below).

Animals and experimental protocol

Mature male Wistar rats (Charles River, Margate, U.K.), five to seven months old, were maintained on a conventional rat pellet diet and water ad libitum and under a controlled normal light–dark cycle throughout the study. Single daily subcutaneous injections of 100 μl of EE or MPA in PG, or PG alone were given without anaesthesia to six rats in each group at 10.00 a.m. for seven days at a dose of 5 mg/kg initial body weight. The rats were anaesthetised (Sagatal, 50 mg/kg intraperitoneally) 24 h after the last injection, and the common bile duct was exposed and cannulated with silicone rubber tubing (18 cm $\times$ 0.25 mm I.D., 0.5 mm O.D.; Esco Rubber, Teddington, U.K.). Rectal temperature was maintained between 37 and 38 °C. The weight of bile collected in the initial 10 min was used to estimate bile volume flow-rate and, after heating at 67 °C for 30 min, the total bile acid concentration was determined with the 3α-HSD method [11] for estimation of bile acid output. Bile was collected for a further 30 min for analysis of individual bile acids by HPLC. In a separate series of experiments it was observed that in normal rats the individual bile acid composition did not change over this period of bile collection, although there was a small decrease (approximately 5%) in bile volume flow-rate. Samples of normal rat bile were obtained via bile duct cannulae from untreated animals under Sagatal anaesthesia. For comparative purposes data are also presented on the effects of bile duct ligation and division, or a sham procedure, on bile acid composition; these groups act as controls for the effect of cholestasis on bile acid composition.

Sample preparation for HPLC

Bile acids in rat bile were extracted into methanol using a C_{18} Sep-Pak cartridge (Millipore, Harlow, U.K.) essentially as described by Whitney and Thaler [12]. This procedure gave 93–99% recovery of total 3α-hydroxy bile acids, as determined enzymatically with 3α-HSD, when 8.7–43.4 μmol of bile acids in 0.5–2.5 ml of bile were applied to the Sep-Pak cartridges. The recovery of trace amounts of [^{14}C]glycocholic acid added to bile was 98.4 $\pm$ 1.1% (mean $\pm$ S.D., $n=7$). The relatively polar triketo bile acid, taurodehydrocholic acid, which eluted from the HPLC column before tauro-β-muricholic acid, and all the other keto bile acids studied were also efficiently extracted by the Sep-Pak procedure (>98%). The methanol extracts were dried under nitrogen reconstituted in mobile phase and filtered by centrifugation (1000 g) through 0.45-μm Nylon-66 microfilters using the Bioanalytical Systems MFI microfiltration system (Scotlab Instruments Sales, Bellshill, U.K.) prior to HPLC. The recovery of [^{14}C]glycocholic acid added to Sep-Pak extracts of bile after filtration through Nylon-66 filters was 98.8 $\pm$ 1.5% (mean $\pm$ S.D., $n=6$). The recovery of endogenous 3α-hydroxy bile acids in rat bile ($n=5$) after Sep-Pak extraction and HPLC analysis was 101 $\pm$ 1.6% and 102 $\pm$ 2.3% (mean $\pm$ S.D.), respectively.

High-performance liquid chromatography

Bile acid standards and bile extracts were analysed using a Waters Assoc. (Millipore, Harlow, U.K.) HPLC system with a Radial-Pak C_{18} (Resolve) 10-μm reversed-phase radial compression column (10 cm $\times$ 8 mm I.D.) with a C_{18} Guard-

Pak precolumn and refractive index detection as previously described [10], except that a mobile phase of methanol–water (65:35, v/v) plus acetic acid (2.5%, v/v) and pH adjustment to 5.15 with 10 *M* sodium hydroxide was required for rat bile acids. Chromatography was continued long enough for unconjugated bile acids, including lithocholic acid, to elute from the column (approximately 60 min) although these bile acids were rarely detected in bile samples in this study.

Concentrations of each of the bile acids in rat bile extracts were obtained by comparison of peak areas with standards using a Trilab 2/Triton 3 on-line computer system and data analysis software (Trivector Systems International, Sandy, U.K.). The latter permitted the use of response factors in the calculation of bile acid concentrations in the bile extracts thereby allowing for differences in detector response of individual bile acids. Response factors were determined automatically by the software from chromatograms of authentic standards run routinely, under identical conditions, with every batch of samples. The detection limit was 5–10 nmol on-column depending on the retention time, and hence the peak height, of the bile acid. Identification of the conjugated bile acids analysed by the above method and present in rat bile was confirmed by: (a) HPLC of bile acids deconjugated with *C. perfringens*; (b) HPLC of the bile acids as dansyl hydrazones [13]; (c) TLC [14,15] of bile acids before and after deconjugation with *C. perfringens* or reduction with 3α-HSD and/or 7α-HSD to produce keto bile acids [16]; (d) TLC of the collected peaks from HPLC. Visualisation of TLC spots was achieved with phosphomolybdate [15] and anisaldehyde [17] spray reagents.

Analysis of data

Results are presented as mean ± S.D., and analysed by Student's *t*-test or Wilcoxon's rank sum test as appropriate.

RESULTS

Bile from normal and control (PG) rats consistently contained eight major bile acid peaks (TMC, GMC, TC, GC, TCDC, THDC, TUDC, TDC), although the method did not distinguish the different isomers of muricholic acid. Separation of isomers of muricholic acid required a more complex chromatographic procedure which was unsuitable for the overall routine analysis reported here. However, further HPLC of the TMC peak from pooled normal bile, using a more aqueous mobile phase (60% methanol) and a 5-μm C_{18} radial compression column, revealed that this peak comprised mostly Tβ-MC (86%) with 14% being due to an earlier eluting taurine-conjugated bile acid with both 3α- and 7α-hydroxyl groups in equal proportions. This bile acid was probably Tα-MC. The ω-isomer (3α,6α,7β-hydroxyl substitution) may have been present as a minor component in the Tβ-MC peak. Small amounts of glycine-conjugated dihydroxy bile acids and unconjugated bile acids were observed in some biles from normal and treated rats. Several bile samples also contained a small peak eluting before TMC (see Fig. 3) which was shown by further study to comprise two taurine-conjugated 3α-hydroxy bile acids, one also having a 7α-hydroxyl group. These may be keto bile acids in view of their retention characteristics on HPLC [16].

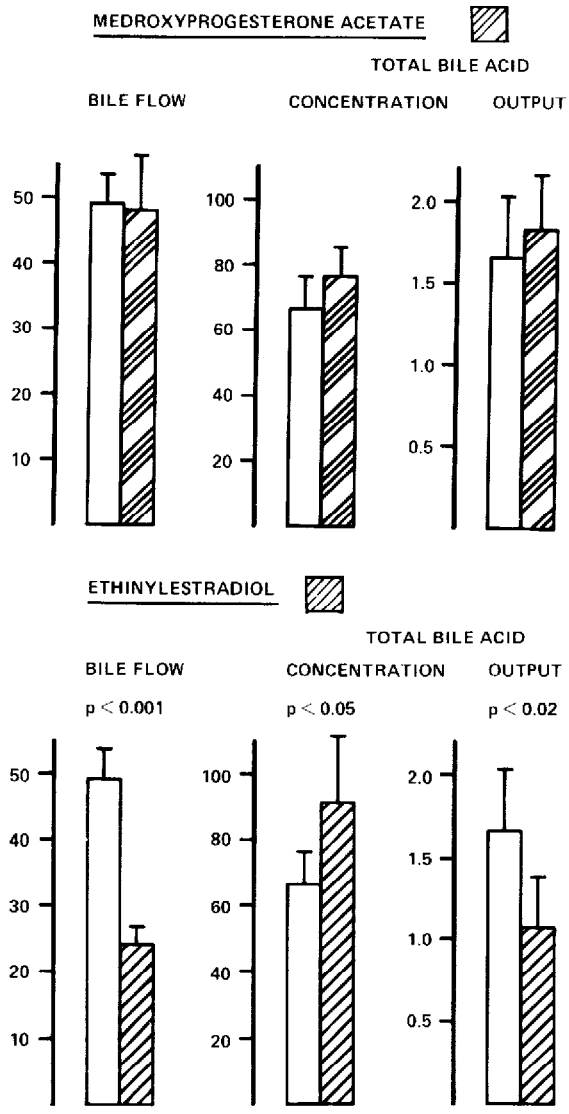

Fig. 1. Bile flow (10 min bile volume flow-rate in mg/min) and total bile acid concentration (μmol/ml) and output (μmol/min) per kg initial body weight in rats given propylene glycol only (open columns) and either medroxyprogesterone acetate (upper) or ethinylestradiol (lower). For each group $n=6$, and the error bars represent 1 S.D.

Chromatography of rat bile before and after the addition of a mixture of standards having the same concentrations as the eight major bile acids (Tβ-MC, Gβ-MC, TC, GC, TCDC, THDC, TUDC, TDC) gave almost identical HPLC profiles. The coefficient of variation (C.V.) obtained from duplicate analysis ($n=12$) of bile from control and steroid-treated rats ranged from 5 to 13% for the six bile acids present in largest amounts, while the two bile acids usually present at less than 1 μmol/ml, TDC and TUDC, were less precisely quantitated (C.V. of 20 and 27%, respectively).

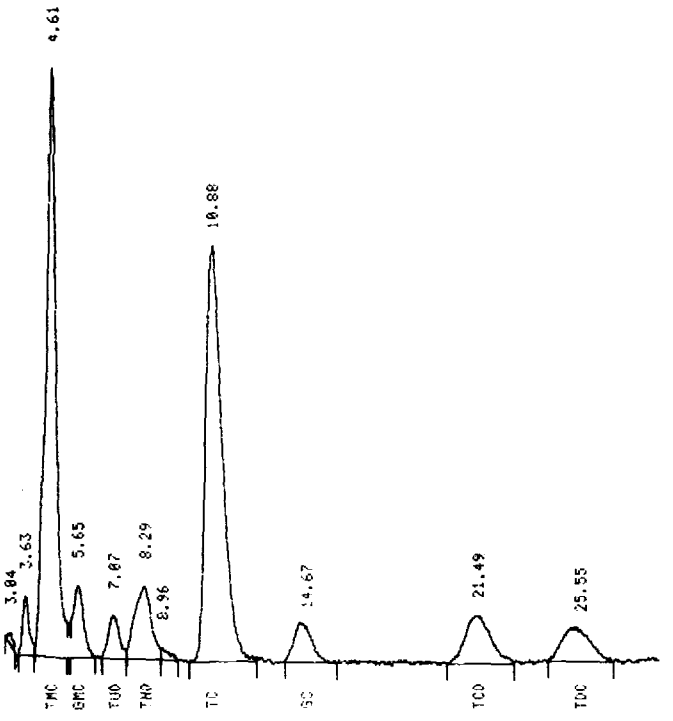

Fig. 2. Individual bile acid profile in bile from a rat given propylene glycol. The peak at 3.63 min represents an unidentified bile acid (see Experimental section). The numbers above the peaks represent the retention times (min), TUD = TUDC, THD = THDC, TCD = TCDC.

Rats given EE or MPA exhibited on average a 7–8% loss in body weight during the seven day treatment (486 ± 13 and 446 ± 18 g and 499 ± 31 and 463 ± 42 g for initial and final body weight of EE and MPA groups, respectively), while injections of PG had little effect (499 ± 39 and 490 ± 48 g).

EE treatment for seven days produced marked cholestasis as evidenced by a decrease of over 50% in the average bile flow in contrast to the normal flow observed in rats given MPA (Fig. 1). Although the total bile acid concentration was increased by EE, the average bile acid output was significantly decreased to 65% of the control value ($p < 0.02$), whereas MPA had little effect (Fig. 1).

The individual bile acid profile in rats given PG was similar to normal (Fig. 2) and in contrast to that observed in rats given EE where conjugated muricholic acids (TMC and GMC) predominated (Fig. 3). Typical bile acid pattern for MPA-treated rats was somewhere between these two. Overall effects of EE and MPA are shown in Fig. 4 which presents each of the eight major bile acids as a percentage of the total. Average changes effected by the steroids were always in the same direction except for TUDC. In both test groups there was a pronounced shift in the average proportions of the major trihydroxy bile acids from conjugated (T plus G) cholic acid (44% in PG, 32% in MPA, 15% in EE) to conjugated muricholic acids (33% in PG, 44% in MPA, 65% in EE). In comparison after seven days of bile duct ligation taurine-conjugated muricholic acid comprised $89 \pm 4\%$ and taurocholic acid $6 \pm 3\%$ ($n = 7$) of the total bile acids in bile collected

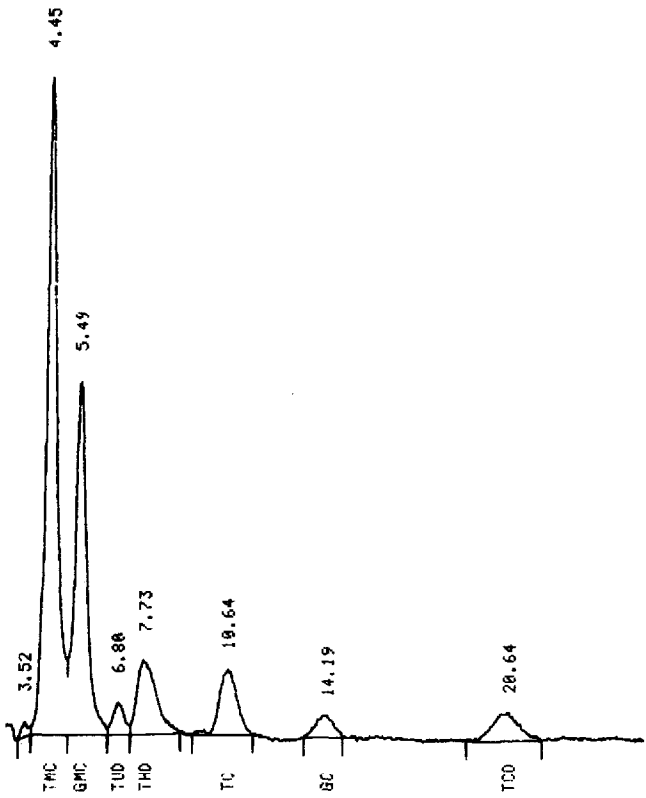

Fig. 3. Individual bile acid profile in bile from a rat given ethinylestradiol. A peak corresponding to TDC was not determined in this example. Other description as for Fig. 2.

by aspiration from the distended bile duct. The corresponding values in sham-operated rats after seven days were 33 ± 7 and $39 \pm 15\%$ ($n=3$) which are similar to the proportions found in the PG control animals.

Although not significantly different from rats given PG only, THDC comprised approximately 10% of the total bile acids in both steroid-treated groups while average TCDC and TDC levels were lower than control. TDC levels were very low in EE-treated rats and occasionally undetectable (Fig. 3). When the two steroid groups were compared, all bile acids except THDC and TCDC were significantly different ($p<0.05$ to $p<0.005$), including TUDC ($p<0.01$) which for either group did not differ significantly from control. Glycine-conjugated dihydroxy bile acids and unconjugated bile acids were present in detectable amounts (up to 3%) in some biles from each group studied, with slightly higher values occurring in the steroid-treated rats.

DISCUSSION

The major bile acids observed in rat bile in this study using HPLC analysis are similar to those identified previously with gas chromatographic–mass spectro-

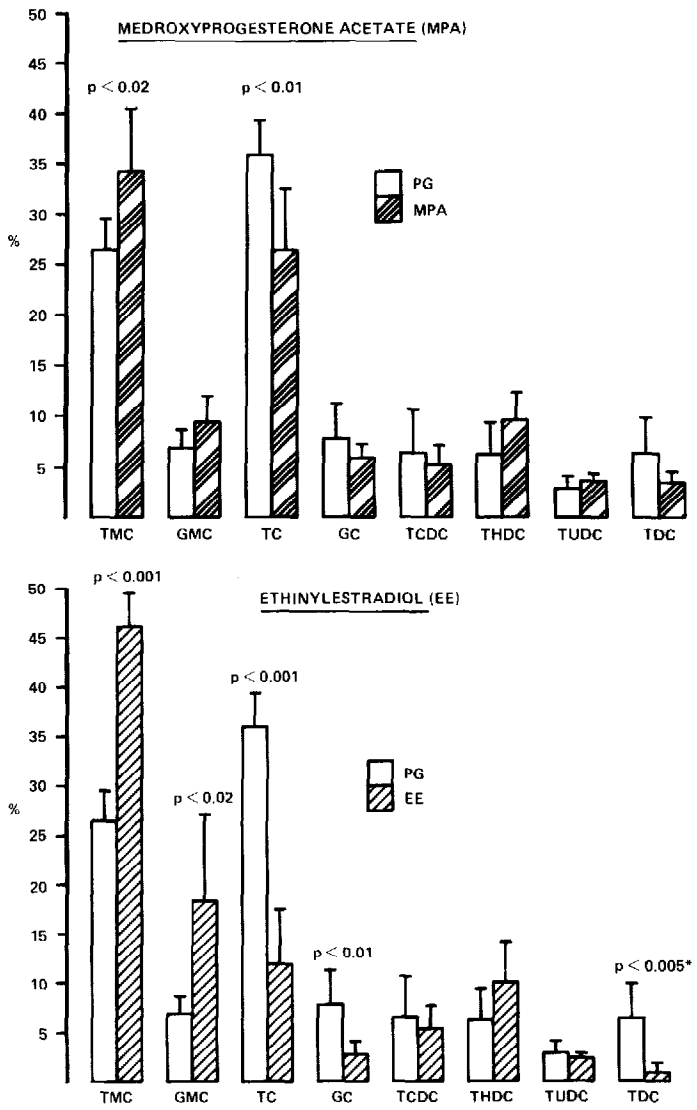

Fig. 4. Percentage individual bile acid composition in rats given propylene glycol only (open columns) and rats treated with medroxyprogesterone acetate (upper) or ethinylestradiol (lower). For each group $n = 6$, and error bars represent 1 S.D. Significant differences are indicated (Student's t-test, except TDC where * indicates Wilcoxon's rank sum test).

metric (GC–MS) techniques [4,18]. Several minor bile acids were also found in rat bile by these workers, including keto and unsaturated bile acids. The glycine/taurine ratios observed with direct analysis using HPLC were only slightly lower than previously obtained by GC–MS analysis of the bile acid classes separated by ion-exchange, which in any event gave poorer recovery of taurine conjugates [18]. Although muricholic acid isomers were not resolved by the routine HPLC method reported here, it is clear that the relative proportion of 3,6,7-trihydroxy bile acids in the bile of rats used in this study is higher, and the cholic

acid proportion lower, than has been observed by other workers using GC–MS methods [4,18]. This may be a consequence of differences in the strain and age of rat, dietary and environmental factors and/or in the analytical techniques employed. A recently reported HPLC method [19], using a post-column immobilised 3α-HSD reactor and fluorescence detection of the NADH produced, appears to give a very similar distribution of the major bile acids in the bile of Wistar rats (after deconjugation) to that found with the method presented here, including the relative proportions of the muricholic acids and cholic acid. In contrast to GC–MS, detection of bile acids by HPLC is relatively non-specific, and overestimation of an individual bile acid due to the presence of other compounds in the peak is a possibility. Unsaturated 3,6,7-trihydroxy bile acids have been reported to occur in rat bile analysed by a combination of TLC and GC–MS techniques [4,18,20,21], and these bile acids may have eluted from the C_{18} HPLC column in the MC peaks. Indeed, unsaturated β-MC derivatives may have accounted for up to 50% of the β-MC fraction or peak obtained by GC or TLC [4,20]. The number of the β-MC derivatives identified in rat bile varies from as many as six [4] to a single major constituent believed to be a β-MC with a double bond in the side-chain [18]. The latter might have accounted for the comparatively low concentrations of β-MC, less than α-MC and CDC, reported by these workers [18]. In an earlier study considerably lower levels of unsaturated β-MC derivatives were observed in bile collected for 12 h, and none were detected in bile collected 48 h after cannulation [21]. These β-MC derivatives appear, therefore, to be products of the intestinal microflora [21] thereby underlining the difficulty of comparing studies on bile acid composition from different laboratories. Nevertheless, the total concentration of the 3,6,7-trihydroxy bile acids identified by GC–MS appears to be lower in relation to the concentration of cholic acid in several studies [4,18,20,21], than was apparently present in the TMC and GMC peaks reported here using the more direct analytical technique of HPLC without deconjugation and derivatization of the bile acids in the original sample. The relative concentrations of THDC and TDC in bile from control and steroid-treated groups lends some support to this observation.

In bile from control rats (PG group) conjugated muricholic acid constituted, on average, 43% of the trihydroxy bile acids, with the taurine conjugate predominating, while TCDC levels were low, confirming the efficiency of 6β-hydroxylation observed with isolated hepatocytes [22,23] and in germ-free rats [24]. These animal models demonstrated that the normal rat liver produces approximately equal amounts of muricholic and cholic acids [22,24] which is consistent with the biliary bile acid composition obtained in the present study in the intact rat.

In the experiments reported here MPA did not produce cholestasis, in terms of reduced bile flow and bile acid output, but did induce changes in the biliary bile acid composition suggesting the steroid may have a direct action on bile acid synthesis, metabolism or secretion. MPA and norethisterone have been reported to exhibit marked effector or repressor actions on microsomal androgen-dependent enzymes of hepatic steroid metabolism [8]. An earlier study had demonstrated that in the male rat norethisterone at low doses stimulated hepatic microsomal 12α-hydroxylation and high doses suppressed 5α-reduction of the

bile acid precursor sterol 7α-hydroxy-4-cholesten-3-one, while 6β-hydroxylase activity on steroid substrates was also increased [25]. However, interpretation of the data obtained from the simple experimental study presented here must be treated with caution. Progestogen-induced changes in bile acid pools, rates of intestinal absorption and bacterial metabolism, which were not studied, may also influence the biliary bile acid profile.

EE at 5 mg/kg per day caused marked cholestasis and had a dramatic effect on the distribution of conjugated muricholic and cholic acids. Others [4] have reported smaller increases in the proportion of muricholic relative to cholic acid in both male and female Sprague-Dawley rats given a lower dose of EE (1 mg/kg per day). Complete cholestasis produced by bile duct ligation resulted in an even greater redistribution of bile acids in favour of TMC. The very high proportion of conjugated muricholic acid in the bile of these rats with ligated bile ducts agrees well with earlier studies of the effects of bile duct ligation on the bile acids in urine and liver using a combination of TLC and GC [20,26]. The increase in the TMC peak is likely to be almost entirely due to β-MC as a result of increased 6β-hydroxylase activity in the liver [20]. Part of the change in biliary bile acid composition in rats treated with EE may therefore be a consequence of cholestasis, although direct action of the estrogen on bile acid metabolism is also likely.

The study in rats reported here indicates that MPA, as well as EE, alters the distribution of individual bile acids in bile although only the latter has cholestatic properties. Some of the changes are due to alterations in the proportions of secondary bile acids, consequent on differences in the relative amounts of the primary bile acids muricholic and cholic acids secreted into the gut, hence the observed levels of taurine conjugates of HDC and DC, the major bacterial products of the primary bile acids [24]. The hepatobiliary consequences of the high doses of synthetic progestogens such as MPA, which are used in the treatment of a number of cancers in both men and women, are largely unknown. Although extrapolation to man is extremely difficult, the observed effects of clinically equivalent doses of MPA on bile acid composition in the rat reported here would seem to warrant further study.

ACKNOWLEDGEMENTS

The author would like to thank Mr. Gary Smith and Mr. Angus Reid for their technical assistance and Mrs. Joyce Mackenzie for preparation of the manuscript. Gary Vitale was in receipt of an American Cancer Society Fellowship No. 6046.

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